Fatty acids play many important roles in cells and also in industrial processes. Furan fatty acids (FuFAs) are present in the lipids of some plant, fish, and microbial species and appear to function as second messengers in pathways that protect cells from membrane-damaging agents. We report here the results of chemical, genetic, and synthetic biology experiments to decipher the biosynthesis of the monomethylated FuFA, methyl 9-(3-methyl-5-pentylfuran-2-yl) nonanoate (9M5-FuFA), and its dimethyl counterpart, methyl 9-(3,4-dimethyl-5-pentylfuran-2-yl) nonanoate (9D5-FuFA), in two α-proteobacteria. Each of the steps in FuFA biosynthesis occurs on pre-existing phospholipid fatty acid chains, and we identified pathway intermediates and the gene products that catalyze 9M5-FuFA and 9D5-FuFA synthesis in *Rhodobacter sphaeroides* 2.4.1 and *Rhodopseudomonas palustris* CGA009. One previously unknown pathway intermediate was a methylated diunsaturated fatty acid, (10E,12E)-11-methyloctadeca-10,12-dienoic acid (11Me-10,12:2), produced from (11E)-methyloctadeca-11-enolic acid (11Me-12E-18:1) by a newly identified fatty acid desaturase, UfaD. We also show that molecular oxygen (O₂) is the source of the oxygen atom in the furan ring of 9M5-FuFA, and our findings predict that an O₂-derived oxygen atom is incorporated into 9M5-FuFA via a protein, UfaO, that uses the 11Me-12E-18:2 fatty acid phospholipid chain as a substrate. We discovered that *R. palustris* also contains a SAM-dependent methylase, FuFM, that produces 9D5-FuFA from 9M5-FuFA. These results uncover the biochemical sequence of intermediates in a bacterial pathway for 9M5-FuFA and 9D5-FuFA biosynthesis and suggest the existence of homologs of the enzymes identified here that could function in FuFA biosynthesis in other organisms.

Fatty acids have numerous cellular and biotechnological functions. In biological membranes, fatty acids form and stabilize the hydrophobic component of the bilayer, act as a permeability barrier, influence the activity of integral membrane proteins, and function as secondary messengers in signaling pathways (1–3). Changes in membrane fatty acid composition often help cells maintain viability in response to temperature and environmental changes and protect them from damage caused by many membrane-active agents (2–8). Industrially, fatty acids or compounds derived from them can serve as food additives, antioxidants, anti-inflammatory compounds, lubricants, or substitutes for other compounds that are typically derived from petroleum (9–12). There is significant potential to produce natural or modified fatty acids through synthetic biology or cellular engineering, given the range of known acyl chains and the number of gene products predicted to produce or modify these compounds across the phylogeny. We are studying the biosynthetic pathway for an unusual yet important fatty acid, a 19-carbon monomethylated furan fatty acid, 9-(3-methyl-5-pentylfuran-2-yl)-nonanoic acid, alternatively named 10,13-epoxy-11-methyl-octadecadienoic acid (9M5-FuFA) (13, 14).

9M5-FuFA is one of a chemically diverse set of furan fatty acids (FuFAs) found in the lipids of select plants, fish, and microbes (15). FuFAs containing zero, one, or two methyl groups are implicated as second messengers in pathways that protect cells from the toxic effects of membrane-damaging agents (15–17). 9M5-FuFA rapidly disappears when cells are exposed to singlet oxygen (15O₂), and this reactive oxygen species is bacteriocidal to cells unable to make this FuFA, illustrating the ability of this FuFA to act as an antioxidant and protect cells from the negative effects of toxic compounds (18). The oxygen atom within FuFAs also provides a site for chemical modifications that could increase their industrial value as lubricants, fuel additives, or biofuels (15). However, strategies to produce large quantities of natural or modified FuFAs are limited by a lack of information about their biosynthesis.

An opportunity to dissect the pathway for FuFA biosynthesis was provided by the increased abundance of 9M5-FuFA fatty acyl chains in phospholipids (18) that are found in a mutant of *Rhodobacter sphaeroides* 2.4.1 with increased expression of genes that respond to 1O₂ (5, 19, 20). Previous reports indicate that a 1O₂-inducible protein RSP2144, UfaM, was necessary for 9M5-FuFA synthesis (18, 21). UfaM is a SAM-dependent methylase that synthesizes a methylated trans-unsaturated fatty acid, methyl (E)-11-methyloctadeca-12-enoate (11Me-12E-18:1), from the cis-vaccenic acid side chains within phospholipids (Fig. 1). Although 11Me-12E-18:1 is a potential intermediate in 9M5-FuFA synthesis, information is lacking on any additional intermediates, the remaining enzyme(s) in this biosynthetic pathway, and other compounds that are needed to produce 9M5-FuFA.
In this work, a methylated diunsaturated fatty acid, (10E,12E)-11-methyloctadeca-10,12-dienoic acid (11Me-10,12-18:1), is identified as another intermediate in 9M5-FuFA biosynthesis. Also identified are previously unknown fatty acid-modifying enzymes from two alpha-proteobacteria (Rhodobacter sphaeroides 2.4.1 and Rhodopseudomonas palustris CGA009) that synthesize 11Me-10,12-18:2 from 11Me-12-18:1 and convert the methylated diunsaturated fatty acid into 9M5-FuFA (Fig. 1). This work also shows that atmospheric oxygen (O_2) is the source of the oxygen atom in the furan ring of 9M5-FuFA. We further show that R. palustris produces a methyl 9-(3,4-dimethyl-5-pentylfuran-2-yl) (9D5-FuFA), which is the first published report of the synthesis of this fatty acyl chain in bacteria, and that a newly discovered protein acts as a SAM-dependent fatty acid methylase to synthesize diunsaturated 9D5-FuFA from the monounsaturated 9M5-FuFA (Fig. 1). These studies provide important new insights into the biosynthesis of furan rings and information to predict the presence of similar biosynthetic pathways and identify genes that can be used to engineer increased production of FuFAs in prokaryotes and eukaryotes.

**Results**

**Genes needed for 9M5-FuFA synthesis**

Deciphering the FuFA biosynthetic pathway is aided by the knowledge of genes needed for the production of 9M5-FuFA in R. sphaeroides (18). For example, fatty acid methyl esters (FAMEs) prepared from the phospholipids found in R. sphaeroides ΔchrR cells contain higher detectable levels of 9M5-FuFA and 11Me-12t-18:1 than WT cells, in addition to the 18:1, 18:0, 16:1, and 16:0 fatty acid chains found in WT cells (Fig. 2A). Previous work (18) also showed that loss of UfaM, which catalyzes the SAM-dependent methylation of cis-vaccenate (18:1), blocked the accumulation of both 9M5-FuFA and 11Me-12t-18:1 (Fig. 2B). In addition, a mutation that deleted all five genes in the RSP1087-1091 operon prevented the accumulation of 9M5-FuFA and led to increased production of 11Me-12t-18:1 (18). From these observations, 11Me-12t-18:1 was proposed to be an intermediate in 9M5-FuFA synthesis, and one or more products of the RSP1087-1091 operon were potential catalysts of subsequent reactions in 9M5-FuFA synthesis (18).

To test these proposals, the fatty acid composition of phospholipids from mutants lacking both chrR (which leads to higher levels of 9M5-FuFA accumulation than in WT cells) and single genes in the RSP1087-1091 operon were analyzed. Examination of this set of isogenic strains showed that placing individual in-frame deletions of either RSP1087, RSP1088, or RSP1089 in ΔchrR cells had no detectable impact on the fatty acid profile compared with cells lacking only chrR (Fig. 2, E–G). This demonstrates that each of these three gene products were not necessary for synthesis of either 9M5-FuFA or 11Me-12t-18:1.

In contrast, inactivation of either RSP1091 (ufaD) or RSP1090 (ufaO) in ΔchrR cells did alter the fatty acid profile compared with that found in cells lacking only the chrR gene (Fig. 2, C and D). Loss of ufaD led to the disappearance of both fatty acids 9M5-FuFA and the previously identified 11Me-12t-18:1. These data predict that both ufaD and ufaO are involved in conversion of 11Me-12t-18:1 into 9M5-FuFA. One possibility is that UfaD converts the previously identified 11Me-12t-18:1 into the fatty acid that is derivatized to the unknown FAME and that UfaO catalyzes the conversion of this compound into the furan-containing fatty acid, 9M5-FuFA (Fig. 1).

To test this hypothesis, the chemical identity of the unknown fatty acid that accumulates in cells lacking ufaO needed to be determined. MS analysis of the FAME derivative indicates that it has a molecular mass of 308.2 Da, but analysis of its fragmentation pattern was insufficient to unambiguously elucidate its
chemical structure. Hydrogenation of the lipids from cells lacking ufaO led to a shift in GC retention time of this FAME (Fig. 3, A and B) and an increase in the parent ion mass by 4 Da to 312.2 Da (Fig. 3, C and D). The magnitude of the mass increase after hydrogenation indicates that the untreated FAME contains two double bonds. The new GC retention time and mass were indistinguishable from those of the hydrogenated form of an 11Me-12t-18:1 FAME (Fig. 3B). These observations suggest that the unknown fatty acid is a diunsaturated derivative of 11Me-12t-18:1.

To determine the chemical identity of this unknown fatty acid, several additional experiments were conducted. A 4,4-dimethyloxazoline (DMOX) derivative of phospholipids from cells lacking ufaO identified a product whose molecular mass (347.3182 Da) predicted the chemical formula (C23H41O1N1), and the fragmentation pattern of the DMOX-modified unknown compound provided additional support for the assignment of the parent fatty acid as a diunsaturated derivative of the monounsaturated 11Me-12t-18:1 (Fig. 4A). These observations suggest that the unknown fatty acid is a diunsaturated derivative of 11Me-12t-18:1.

Conversion of 11Me-12t-18:1 to 11Me-10t,12t-18:2 and 9M5-FuFA

The above results predict that the FuFA biosynthetic pathway utilizes three gene products (ufaM, ufaD, and ufaO) that sequentially convert cis-vaccinate (18:1(11E)) into 11Me-12t-18:1, 11Me-10t,12t-18:2, and 9M5-FuFA (Fig. 1). To test this hypothesis, FAME profiles of phospholipids prepared from cells containing different combinations of ufaM, ufaD, and ufaO cloned behind an isopropyl β-D-1-thiogalactopyranoside (IPTG)-inducible promoter on a low-copy plasmid were examined. The parent strain for this study contained an intact chrR gene but lacked all three of these genes (ufaM, ufaD, and ufaO) on the chromosome. Use of this strain allowed us to separate the synthesis of 9M5-FuFA and its putative biosynthetic intermediates from the increases in expression of other genes that are caused by the loss of chrR (Fig. 5A) (23, 24). FAMEs of phospholipids from the parent strain (missing ufaM, ufaD, and ufaO) on the chromosome lacked detectable levels of 9M5-FuFA or any of the putative FuFA pathway intermediates (Fig. 5B). In contrast, when this parent strain contains only the ufaM gene on a plasmid, it accumulated a fatty acid corresponding to 11Me-12t-18:1 (Fig. 5C), as predicted from its known activity as a fatty acyl methylase (18). In addition, fatty acids isolated from this parent strain containing ufaM plus ufaD on the same plasmid produced 11Me-10t,12t-18:2 (Fig. 5D), whereas fatty acids isolated from the same strain

Figure 2. The conversion of 11Me-12t-18:1 into 9M5-FuFA requires both RSP1090 (UfaO) and RSP1091 (UfaD). Above the panels is a representation of the genes within the R. sphaeroides RSP1091-1087 operon. A–G, the GC elution profiles of FAMEs generated from ΔchrR cells (A) as well as ΔchrR cells that also contain a ufaM deletion (B) or contain additional in-frame deletions in ufaD (RSP1091) (C), ufaO (RSP1090) (D), RSP1089 (E), RSP1088 (F), and RSP1087 (G).
containing ufaM plus ufaD and ufaO on this plasmid accumulated 9M5-FuFA (Fig. 5D). These results predict that these three genes are sufficient in vivo for 9M5-FuFA synthesis from cis-vaccenic acid (18:1), the fatty acid substrate for the SAM-dependent methylase, UfaM (18).

**11Me-10t, 12t-18:2 is a product of desaturation of 11Me-12t-18:1**

The results of the above experiments further predict that 11Me-10t, 12t-18:2 is converted from 11Me-12t-18:1 by UfaD. To test this hypothesis, a recombinant His8-tagged version of UfaD was incubated with *R. sphaeroides* phospholipid-derived liposomes and tested for fatty acyl modifications. When assays were performed in the presence of liposomes that contained 11Me-10t, 12t-18:2 fatty acids (prepared from a strain lacking ufaD), the predicted fatty acid substrate for desaturation, a FAME with a GC retention time and MS fragmentation pattern identical to methyl 11Me-10t, 12t-18:2 was detected (Fig. 6, A and B). All of the detected 18:2 fatty acid products were methylated, suggesting that UfaD did not desaturate the abundant 18:1 fatty acid found in *R. sphaeroides* phospholipids. The results of this experiment allow us to contend that UfaD is a previously unknown fatty acyl desaturase and that this protein has a high degree of specificity for 11Me-12t-18:1 as a substrate.
EDITORS' PICK: Methylated furan fatty acid biosynthesis

A

Formula mass (C₉H₁₀NO₅): 347.3183 Da
C₁₀H₁₀NO₅ 250.217 Da
C₁₁H₁₀NO₅ 262.217 Da
C₁₂H₁₀NO₅ 274.217 Da
C₁₃H₁₀NO₅ 286.217 Da

B

(J₁₂⁻H₁3) = 15.6 Hz = E

11Me-10t, 12t-18:2
The predicted product of UfaD activity, 11Me-10\(\text{t}\),12\(\text{t}\)-18:2, accumulates when \(\Delta\text{chrR}\) cells also lacking ufaO are grown in the presence of \(\text{O}_2\) (Fig. 2D). However, 11Me-10\(\text{t}\),12\(\text{t}\)-18:2 does not accumulate to detectable levels when the same strain was grown in the absence of \(\text{O}_2\) (Fig. S1). Thus, \(\text{O}_2\) is required for 11Me-10\(\text{t}\),12\(\text{t}\)-18:2 formation, possibly because it serves as an electron acceptor for the newly identified UfaD desaturase (25).

\(\text{O}_2\) is the source of the oxygen atom in the furan ring of 9M5-FuFA

Another prediction from the above data are that 9M5-FuFA is derived from 11Me-10\(\text{t}\),12\(\text{t}\)-18:2. When a recombinant His6-UfaO protein synthesized in vitro using a wheat germ cell-free extract was incubated with \(\text{R. sphaeroides}\) liposomes containing 11Me-10\(\text{t}\),12\(\text{t}\)-18:2 (a possible substrate for UfaO), a FAME with a GC retention time and MS profile that matched 9M5-FuFA was observed.

The assembly of the unknown 11Me-10\(\text{t}\),12\(\text{t}\)-18:2 diunsaturated fatty acid that accumulates in \(\Delta\text{chrR}\Delta\text{ufaD}\) cells. A, MS fragmentation of a DMOX derivative of the fatty acid that is accumulated in \(\Delta\text{chrR}\Delta\text{ufaO}\) cells. The diagnostic fragmentation products that identify this as a DMOX derivative of an 11Me-10\(\text{t}\),12\(\text{t}\)-18:2 fatty acid are labeled \(a\)–\(d\) on the structure and mass spectrum. B, 2D NOE \(\text{H}^1\text{H}^1\) correlation spectra of the FAME. The NOE (through-space) correlation peaks indicate the presence of one dominant geometrical isomer (10\(\text{t}\),12\(\text{t}\)), as was also deduced from GC-MS.

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To test the source of the oxygen atom in the furan ring of 9M5-FuFA, this assay was repeated under an atmosphere of 18O2 (99% isotopic enrichment). In these reaction products, the diagnostic M\(^{+}\), \(a\)-ion (167.1 Da), and \(b\)-ion (111.1 Da) derived from 9M5-FuFA each had a mass that was ~2 Da larger than the ones present when the reaction was performed in the presence of air (322.2, 165.1, and 109.1 Da, respectively, Fig. 8 (A and B)). The observed 2-Da increase in the mass of these ions and their abundance compared with their counterparts when a control reaction was performed in 16O2 atmosphere shows that O2 is the source of the oxygen atom in the furan ring of 9M5-FuFA.

As an independent test of the source of the oxygen atom in 9M5-FuFA, the fatty acid profile of ΔchrR cells grown aerobically in the presence of either 16O\(_2\) (Fig. 8C) or 18O\(_2\) (Fig. 8D) were analyzed. When cells are grown in the presence of 18O\(_2\), the diagnostic M\(^{+}\), \(a\)-ion, and \(b\)-ion from 9M5-FuFA FAMEs were 2 Da larger than those found when the same strain is grown in the presence of air. In addition, FAMEs derived from the 9M5-FuFA obtained from cells grown in the presence of 18O\(_2\) have a mass and fragmentation pattern indistinguishable from the product of UfaO activity (Fig. 8B).

Based on the results of the above in vitro and in vivo experiments, O2 is the direct source of the oxygen atom in the furan ring of 9M5-FuFA, suggesting that UfaO is a previously unknown fatty acyl-modifying enzyme that uses the methylated diunsaturated fatty acid, 11Me-10\-_t,12\-_t-18:2, as a substrate. The use of O2 as the source of the oxygen atom in 9M5-FuFA is consistent with the results of previous in vivo
experiments that demonstrated that this FuFA only accumulated in phospholipids when \( \Delta chrR \) cells were grown under aerobic conditions (18).

**The FuFA biosynthetic pathway exists in other bacteria**

Other cells are predicted to contain proteins related to UfaM, UfaD, and UfaO (24). To test this hypothesis, we tested whether the *R. palustris* UfaM, UfaD, and UfaO homologs (RPA2569, RPA2571, and RPA2570, respectively) were sufficient to produce the expected fatty acyl chains. To do this, these three genes were cloned behind an IPTG-inducible promoter on a low-copy plasmid and placed in the *R. sphaeroides* \( \Delta ufaM \), \( \Delta ufaD \), and \( \Delta ufaO \) triple-mutant strain. Analysis of FAMES prepared from phospholipids from this strain showed that these three *R. palustris* genes are sufficient to produce 11Me-12t-18:1, 11Me-10t,12t-18:2, and 9M5-FuFA in *R. sphaeroides* (Fig. 9C) (Fig. 9 (A and B)) shows data from biological replicates of Fig. 5 (A and B)). These data demonstrate that proteins homologous to *R. sphaeroides* UfaM, UfaD, and UfaO are sufficient for production of 9M5-FuFA and its predicted pathway intermediates.

The above finding led us to ask whether *R. palustris* contained intermediates or products of the 9M5-FuFA pathway. It was especially important to test this prediction as published analyses do not report the accumulation of a FuFA (9M5-FuFA or others) in *R. palustris* (26). By analysis of the FAME profile of phospholipids from aerobically grown *R. palustris* cells, we identified 9M5-FuFA and 11Me-12t-18:1 (Fig. 10). The accumulation of these compounds was abolished when cells contained an in-frame deletion of RPA2569 (ufafM), RPA2570 (ufafO), and RPA2571(ufafD), but when these three genes were expressed from a plasmid in the \( \Delta RPA2569-2571 \) mutant, 11Me-12t-18:1 and 9M5-FuFA production was restored (Fig. 10). From these results, we conclude that the RPA2569–2571 gene products are necessary and sufficient for 9M5-FuFA production by *R. palustris*.

**Bacterial synthesis of a dimethyl-FuFA**

The above analysis of *R. palustris* phospholipid FAMES showed that this bacterium contained a product with a molecular mass 336.52 and a MS fragmentation pattern (Figs. 10 and 11) diagnostic of a dimethyl-FuFA, 9D5-FuFA (Fig. 1). Accumulation of the putative 9D5-FuFA is abolished when the RPA2569–2571 genes are inactivated (Fig. 10), suggesting that this compound is synthesized from 9M5-FuFA via a heretofore unidentified FuFA methylase. The *R. palustris* genome contains two genes annotated as SAM-dependent fatty acid–modifying enzymes (RPA3082 and RPA0924) with 38 and 26% amino acid identity, respectively, to the known *R. sphaeroides* fatty acyl methylase UfaM (26).

To test the role of these putative fatty acid–modifying enzymes in the synthesis of 9D5-FuFA, we analyzed the impact of inactivating each of these genes on the phospholipid profile. There were no significant changes in the profile when RPA3082 was deleted (Fig. S2). In contrast, when cells lacked only the RPA0924 (fufM) gene, 9M5-FuFA was the only FuFA produced, implicating this protein in synthesis of 9D5-FuFA. To test this hypothesis, *ufM* was cloned behind an IPTG-inducible promoter and expressed in *R. sphaeroides* \( \Delta chrR \) cells that accumulated 9M5-FuFA (Fig. 2). This *R. sphaeroides* strain accumulated 9D5-FuFA (Fig. 9D). Thus, we predict that *ufM* is sufficient for 9D5-FuFA synthesis, presumably because it methylates 9M5-FuFA. Indeed, when we incubated an *in vitro* synthesized FuF protein with SAM and 9M5-FuFA-containing liposomes (prepared from *R. sphaeroides* \( \Delta chrR \) cells), there was synthesis of a compound with a retention time and MS fragmentation pattern (Figs. 10 and 11) diagnostic of a dimethyl-FuFA, 9D5-FuFA. Based on this, FuM is a previously uncharacterized 9M5-FuFA methylase that produces 9D5-FuFA.

**Discussion**

FuFAs are a broad class of fatty acids with zero, one, or two methyl groups in oxygen-containing (epoxy) rings that are positioned at different places in the acyl chain (13, 15). This work
Figure 10. The FAME profiles produced from *R. palustris* CGA009 and mutant strains. A–I, GC elution profiles of the FAMEs prepared from the indicated *R. palustris* strains containing an in-frame deletion of individual *R. palustris* genes without or with the noted gene on a plasmid. A, WT CGA009; B, ΔRPA2569; C, ΔRPA2569 + ufaM; D, ΔRPA2571; E, ΔRPA 2571 + ufaD; F, ΔRPA2570; G, ΔRPA2570 + ufaO; H, ΔRPA0924; I, ΔRPA0924 + FufM.
describes a previously unknown bacterial pathway for the synthesis of mono- and dimethyl-FuFAs, a poorly characterized group of membrane fatty acids found in microbes, animals, and plants. Despite the known or predicted roles of FuFAs as bilayer components or cellular antioxidants (15), little is known about their biosynthesis (22, 27). Our study identified additional intermediates in FuFA biosynthesis, reports on several previously unknown enzymes involved in mono- and dimethyl-FuFA biosynthesis, and demonstrates a role for O2 in two steps of this pathway. Below, we relate our findings to previous analyses of bacterial FuFA synthesis, describe the chemistry involved in biosynthesis of these fatty acids, and illustrate how our findings can be used to predict the existence of similar pathways in other biological systems.

The FuFA biosynthetic pathway

Previous work showed that *R. sphaeroides* synthesized 9M5-FuFA by modifying pre-existing phospholipid fatty acyl chains (18). Several biosynthetic schemes have been proposed for mono- or dimethyl-FuFA synthesis in prokaryotes and eukaryotes, each of which assumes that these acyl chains are derived from pre-existing fatty acids (13, 14, 22, 27, 28). However, there is no experimental evidence for predicted pathway intermediates, the identity of FuFA biosynthetic enzymes, the source of oxygen in the furan ring, or whether dimethyl-FuFA is synthesized directly from monomethylated derivatives. The only biochemical data on intermediates in FuFA biosynthesis come from studies with *R. sphaeroides*. In this

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**Figure 11. Analysis of FAMEs produced from in vitro incubation of phospholipids with His6-FufM protein.** A, GC elution profiles of FAMEs from lipids in wheat germ cell-free extract. B, FAMEs from phospholipid liposomes prepared from ΔchrR cells. C, FAMEs in which phospholipid liposomes from ΔchrR cells were mixed with His6-FufM protein synthesized using wheat germ cell-free extract. D, FAMEs after the materials in C were incubated with 25 μM SAM. E, mass spectrum of the FAME that elutes at 18.75 min with key diagnostic ions for 9D5-FuFA highlighted.
bacterium, the first step of 9M5-FuFA synthesis is catalyzed by a SAM-dependent fatty acyl methylase (UfaM) that produces a methylated unsaturated fatty acid (11Me-12t-18:1) from cis-vaccenic acid (18). However, it is not known whether 11Me-12t-18:1 directly converts to 9M5-FuFA or if additional intermediates are involved in the biosynthesis of this fatty acid chain; nor are the identity and biochemical properties of other enzymes needed to synthesize this FuFA known. Below, we summarize new insights gained into FuFA biosynthesis by our studies.

A newly identified diunsaturated fatty acid intermediate involved in 9M5-FuFA synthesis

The accumulation of a new fatty acid when ufaO was inactivated suggested that this compound was a potential intermediate in FuFA biosynthesis. However, the identity of the FAME derivative of this fatty acid could not be unambiguously determined by its mass and fragmentation pattern. Instead, GC-MS of a DMOX derivative and NMR analysis of the FAME was used to identify the unknown fatty acyl chain that accumulates when ufaO was inactivated as 11Me-10t,12t-18:2. The DMOX derivative provides a way to distinguish 11Me-10t,12t-18:2 from 2-ocetylcylopropen-1-octanoic acid whose FAME mass spectrum is identical to that of the diunsaturated FAME. Our data show that UfaD is necessary and sufficient for the synthesis of 11Me-10t,12t-18:2 from 11Me-12t-18:1 and that the newly incorporated double bond in this diunsaturated fatty acid is primarily in the trans-configuration.

UfaD is annotated as a fatty acid–modifying enzyme and is predicted to contain a NAD-FAD–binding domain, which has significant amino acid identity to reductases and dehydrogenases (including phytoene dehydrogenases and desaturases) that generate a polyunsaturated intermediate during carotenoid biosynthesis (29). As 11Me-10t,12t-18:2 synthesis in vivo was only detected when cells were grown aerobically (Fig. S1), we propose that UfaD is a newly discovered member of a family of fatty acid desaturases that use O2 as an electron acceptor (25). In addition, the failure to observe conversion of unbranched 18:1 to 18:2 in vitro suggests that UfaD has a substrate preference for the methyl-terminated substrate 11Me-12t-18:1, possibly relating to the strength of the C–H bond that must be broken to proceed with the desaturation reaction. The R. palustris homologue of UfaD, RPA2571, catalyzes the same reaction in vivo, suggesting that the UfaD protein family and its role in FuFA synthesis is conserved in other bacteria. Further information on the specificity for acyl chain and desaturation site and the requirement for O2 and other cofactor(s) for UfaD activity, requires purification and detailed biochemical characterization of this enzyme.

**O2 is the source of the oxygen atom in 9M5-FuFA**

FuFAs, such as 9M5-FuFA, are one of the few classes of natural fatty acids with an oxygen atom within the hydrophobic portion of the acyl chain. Hydroxylases, lipoxygenases, or other enzymes have been proposed or shown to incorporate an oxygen atom into the furan ring of terpenoids, antimicrobials, and other compounds (14, 22, 30–32). The source of the oxygen atom in the FuFA rings is O2; however, the enzyme(s) involved is unknown (33, 34).

Homologs of cytochrome P450 monooxygenase act on fatty acid substrates, and fatty acyl monooxygenases are known to be involved in acyl chain metabolism (35). This work demonstrates that O2 is the source of the oxygen atom in 9M5-FuFA and that UfaO is necessary and sufficient for the synthesis of this FuFA from 11Me-10t,12t-18:2. This report identifies a potential new class of fatty acyl monooxygenases, UfaO, that use methylated polyunsaturated fatty acids as substrates and directly incorporates O2 to form FuFAs. A related protein from R. palustris (RPA2570) is required for, and catalyzes, the same step in FuFA synthesis, making it the second reported member of the UfaO protein family.

UfaO homologs are found in many bacteria (24) and are not yet annotated or reported with an enzymatic function. However, they contain a domain of unknown function, DUF1365, that is broadly distributed in Bacteria and Eukarya (36). Thus, it is possible that additional members of the DUF1365 protein family are also here to perform uncharacterized UfaO homologues, or that DUF1365 has a presently unknown role in oxidation reactions. Monoxygenases typically require a reductant for catalysis, and the one used by UfaO is currently unknown. Synthesis of 9M5-FuFA from 11Me-10t,12t-18:2 and O2 did not require the addition of an exogenous reductant, suggesting that sufficient levels of a potential reductant were present in the wheat germ extracts used to assay this reaction in vitro. Details on the cofactor(s) and the molecular mechanism of this newly identified FuFA biosynthesis enzyme awaits the progress of ongoing efforts to purify and characterize UfaO proteins that are active with 11Me-10t,12t-18:2 as a substrate.

**The dimethyl-FuFA, 9D5-FuFA is synthesized from 9M5-FuFA**

We also report on the presence and biosynthesis of the dimethyl-FuFA, 9D5-FuFA, in the α-proteobacterium, R. palustris. We show that 9D5-FuFA is synthesized from 9M5-FuFA in both the native host and in another bacterium that is engineered to contain the needed enzymes, and we identify FuFM (RPA0924) as a SAM-dependent methylase in vitro and in vivo. When active His<sub>6</sub>-FuFM enzyme preparations are incubated in the presence of 18:1 (11Z) fatty acids, no significant methylation of this substrate is detected, illustrating the relative specificity of FuFM for methylating 9M5-FuFA. Comparing the acyl chain specificity of FuFM with that of other SAM-dependent fatty acyl methylases (UfaM, cyclopropane fatty acid synthase, etc.) provides an opportunity to understand the molecular basis for hydrocarbon substrate specificity. This work also provides methods and strains to determine whether other FuFM homologs produce dimethyl-FuFAs.

**Conclusion**

In sum, this work reports new information on the biosynthetic pathway and intermediates in the synthesis of two FuFAs (9M5-FuFA and 9D5-FuFA) and gene products (UfaD, UfaO, and FuFM) involved in the production of these FuFAs. Both mono- and dimethyl-FuFAs are synthesized from 18:1 fatty acyl chains on phospholipids, and 9D5-FuFA is synthesized...
directly from 9M5-FuFA by a previously undescribed SAM-dependent fatty acid methylase, FufM. Homologues of the enzymes involved in monomethyl FuFA synthesis are present across the bacterial phylogeny (24) and in eukaryotes, so it is possible that many other organisms use similar chemical intermediates in a previously undescribed biosynthetic pathway to produce FuFAs. In contrast, FufM homologs are found only in a subset of the organisms that are predicted to contain UfaM, UfaD, and UfaO homologues, suggesting that 9D5-FuFA synthesis is not as widespread as 9M5-FuFA production. The ufaD, and ufaO homologues, suggesting that 9D5-FuFA synthesis is not as widespread as 9M5-FuFA production. The knowledge gained from these studies helps us to understand the chemical transitions needed for FuFA biosynthesis. It also provides the information needed to test for cofactors needed for synthesis of FuFAs and to begin the biochemical analysis of the enzymes that act in this biosynthetic pathway. Further, our findings provide biochemical approaches to over-produce FuFAs for industrial use and understand their role as membrane second messengers and antioxidants (18, 21).

**Experimental procedures**

**Bacterial strains and growth**

*Escherichia coli*, *R. sphaeroides*, and *R. palustris* strains were grown as described (18, 37) with the exception that when *R. palustris* was grown for phospholipid analysis, it was grown in pMACY-SCAV medium, which replaces yeast extract with 5 g/l. *R. palustris* was grown for phospholipid analysis, it was grown in pMACY-SCAV medium, which replaces yeast extract with 5 g/ml casamino acid (37). When necessary, media were supplemented with 20 μg/ml gentamycin, 50 μg/ml kanamycin, 20 μg/ml chloramphenicol, or 10% sucrose (w/v).

**Gene cloning**

Mutant strains and complementation plasmids (Table 1) were made using methods described previously (21, 49) and by using the NEBuilder HiFi DNA Assembly cloning kit (New England Biolabs) (Table S1). Mini-prep kits for cloning were from Zymogen; maxi-prep kits were from Omigene. See Table S1.

**FAME analysis**

Samples were prepared and analyzed as described previously (18) with the exception that they were kept away from light to minimize fatty acid photooxidation and photoisomerization, all solutions were degassed with N2, and FAMEs were resuspended in 250 μl of hexane for analysis by GC-MS.

**Hydrogenation and identification of unknown FAME**

FAMEs were created as described previously (18) from 13 ml of aerobically grown ΔchrΔufaO cells and dried down under N2. This material was resuspended in 3 ml of dichloromethane in a round-bottom flask, degassed with H2 prior to the addition of 2 mg of 5% palladium on activated charcoal (Sigma), and stirred. The sample was sparged with H2 in a balloon and incubated for 2 h at room temperature and processed as described above for GC-MS studies. Preparation of the DMOX (4,4-dimethyloxazoline) derivatives was as described (50) with the exception that the reactions were incubated at 190 °C for 18 h; extracted by adding 5 ml of diethyl ether/hexane (1:1), 5 ml of water, and −0.3 g of NaCl; and dried with sodium sulfate and N2. The products were reconstituted in 100 μl hexane, and 1-μl aliquots (split 1:10) were analyzed on a Thermo Scientific Q Exactive GC Orbitrap GC-MS/MS system equipped with a Trace 1310 GC and TriPlus RSH autosampler using the following oven program: 150 °C isothermal for 4 min, 6 °C/min ramp to 245 °C, and then 80 °C/min ramp to 325 °C and isothermal at 325 °C for 2 min. The MS transfer line was heated to 300 °C, the MS source was held at 250 °C, and the ionization energy was 70 eV. The GC inlet temperature was set to 275 °C, analyzer mass resolution was set to 70,000, and the automatic gain control target and maximum ion times were set to automatic. Spectral interpretation and elucidation of the location of double bonds by the mass spectra of DMOX derivatives is described in (51–56) (Christie, W. W. (2017) Mass Spectrometry of Dimethyloxazoline and Pyrrolidine Derivatives Epoxy, Furano and Alkoxyl Fatty Acids; https://www.lipidhome.co.uk/ms/dmox/dmox-epoxy/index.htm; Accessed July 13, 2017).

**Isolation of ΔchrΔufaO phospholipids and FAME creation for NMR analysis**

FAMEs were created as described above from 1.6 × 10^{12} ΔchrΔufaO cells. The crude mixture of FAMEs was purified using HPLC. Briefly, the mixture of FAMEs was dissolved in acetonitrile (2 ml) and filtered through a 0.2-μm PTFE syringe filter. The sample was then injected onto a Shimadzu LC-20 equipped with a Phenomenex Luna 5-μm C18(2), 100 Å, 250 × 10-mm column held at 50 °C and a photodiode array detector (Shimadzu, SPD-M20). The mobile phase was a binary gradient of acetonitrile and water that ramped from 20% acetonitrile to 100% acetonitrile over 35 min. Fractions were collected by hand and were selected based on the absorption chromatogram at λ = 300 nm. Each fraction was dried under nitrogen, and FAME identity and purity was determined using an Agilent 7890A/5975C GC-MS as described previously (18).

**Ectopic expression of FuFA biosynthetic genes**

Plasmid pIND5 was used for expression in *R. sphaeroides* and pBBR1mcs5 for expression in *R. palustris*. Triplet biological cultures were separately treated with 1 mM IPTG (for *R. sphaeroides*) and pVPufaD, was inoculated into autoinducing ZMS-dc medium, which replaces yeast extract with 5 g/ml kanamycin, 20 μg/ml chloramphenicol, or 10% sucrose (w/v).

**Purification of His_{6}-UfaD protein**

A 500-ml culture of log-phase B834 *E. coli* cells (40, 41), containing pVPufaD, was inoculated into autoinducing ZMS-80155 (57) medium containing plasmid pRARE2 (Novagen) to induce expression of N-terminally octahistidine-tagged protein (His_{6}-UfaD), with shaking for ~24 h at room temperature (58). The cells were harvested by centrifugation, and the resulting pellet was resuspended in lysis buffer (50 mM NaOHPO, pH 7.5, 1 M NaCl, 20 mM imidazole, 10% Sodium Lauroyl Sarcosinate, and 1× Halt protease inhibitor (Pierce), sonicated on ice, pulsing every 30 s, for 20 min, and centrifuged for 1 h at 20,000 X g. The resulting supernatant was incubated with 4 ml of Ni-
**EDITORS’ PICK: Methylated furan fatty acid biosynthesis**

### Table 1

#### Strains and plasmids

| Strains          | Relevant genotype                                                                 | Source/Reference       |
|------------------|-----------------------------------------------------------------------------------|------------------------|
| E. coli          |                                                                                  |                        |
| DH150           | supE44 lacI169Q80 lacZ2 M15 hisD178 recA1 endA1 gyrA96 thi-1 relA-1                | Ref. 38                |
| 5-alpha         | F’/endA1 hisD178 (R’K’ mk’ M’ ) glnV44 thi-1 recA1 gyrA (NalR) relA1 Δ(lacZYA-argF)U169 deor (de80lacΔ(lacZ)M15) | New England Biolabs    |
| S17-1           | C600::RP-4-2-(Tc::Mu) (Kc:Trn7) thi pro hisD Rsd M’ recA                           | Ref. 39                |
| BS34           | F ompT I16899 (rpsI mbb) gld dcm met (DE3)                                         | Refs. 40–42            |
| R. sphaeroides  |                                                                                  |                        |
| RPA0923         |                                                                                  |                        |
| RPA0923mob      |                                                                                  |                        |
| RPA1091         |                                                                                  |                        |
| RPA1090         |                                                                                  |                        |
| RPA0924         |                                                                                  |                        |
| CGA009          |                                                                                  |                        |
| CGA009ΔRPA2569  |                                                                                  |                        |
| CGA009ΔRPA2570  |                                                                                  |                        |
| CGA009ΔRPA2571  |                                                                                  |                        |
| CGA009ΔRPA2572  |                                                                                  |                        |
| CGA009ΔRPA0923  |                                                                                  |                        |
| CGA009ΔRPA0924  |                                                                                  |                        |
| CGA009ΔRPA0924ΔRPA0923 |                                                                                 |                        |
| CGA009ΔRPA3082  |                                                                                  |                        |
| CGA009ΔRPA324  |                                                                                  |                        |
| R. palustris     |                                                                                  |                        |
| CGA009          |                                                                                  |                        |
| CGA009ΔRPA2569  |                                                                                  |                        |
| CGA009ΔRPA2570  |                                                                                  |                        |
| CGA009ΔRPA2571  |                                                                                  |                        |
| CGA009ΔRPA2572  |                                                                                  |                        |
| CGA009ΔRPA0923  |                                                                                  |                        |
| CGA009ΔRPA0924  |                                                                                  |                        |
| CGA009ΔRPA0924ΔRPA0923 |                                                                                 |                        |
| CGA009ΔRPA3082  |                                                                                  |                        |
| CGA009ΔRPA324  |                                                                                  |                        |

#### Plasmids

| Plasmids          | Relevant genotype                                                                 | Source/Reference       |
|-------------------|-----------------------------------------------------------------------------------|------------------------|
| pK18mobsacB       | oriV oriT mob sacB Kn’                                                              | Ref. 45                |
| pRSBY1            | 6.6-kb fragment containing RSP1091–RSP1087 operon                                   | Ref. 21                |
| pKΔ1087mob        | Into the XbaI/HindIII site of pK18mobsacB − 1057 bp upstream and + 1068 bp downstream of RSP1087 while deleting 723 bp (of 738 bp) in RSP1087. Deletes 241 of the 245 amino acids of RSP1087. | This study            |
| pKΔ1088mob        | Into the XbaI/HindIII site of pK18mobsacB − 1043 bp upstream and + 1104 bp downstream of RSP1088 while deleting 510 bp (of 561 bp) in RSP1088. Deletes 170 of the 186 amino acids of RSP1088. | This study            |
| pKΔ1098mob        | Into the XbaI/HindIII site of pK18mobsacB − 1066 bp upstream and + 1104 bp downstream of RSP1089 while deleting 1161 bp (of 1206 bp) in RSP1089. Deletes 387 of the 402 amino acids of RSP1089. | This study            |
| pKΔ1099mob        | Into the XbaI/HindIII site of pK18mobsacB − 1032 bp upstream and + 1002 bp downstream of RSP1090 stop while deleting 708 bp (of 750 bp) in RSP1090. Deletes 212 of the 250 amino acids of RSP1089. Potential promoter of RSP1089 is kept. | This study            |
| pKΔ9190mob        | Into the XbaI/HindIII site of pK18mobsacB − 745 bp upstream RSP1091 and + 1265 bp downstream of RSP1090 stop while deleting 708 bp (of 750 bp) in RSP1090. Deletes all amino acids of 1091 and 1090. | This study            |
| pKΔ2569mob        | −1012 bp upstream and + 1026 bp downstream of RPA2569 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA2569. | This study            |
| pKΔ2570mob        | −1030 bp upstream and + 1048 bp downstream of RPA2570 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA2570. | This study            |
| pKΔ2571mob        | −1034 bp upstream and + 1048 bp downstream of RPA2571 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA2571. | This study            |
| pΔ0923mob         | −1030 bp upstream and + 1030 bp downstream of RPA0923 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA0923. | This study            |
| pΔ0924mob         | −1030 bp upstream and + 1030 bp downstream of RPA0924 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA0924. | This study            |
| pΔ2324mob         | −1530 bp upstream of RPA0923 and + 1530 bp downstream of RPA0924 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA0923 and RPA0924. | This study            |
| pΔ0928mob         | −1025 bp upstream and + 1000 bp downstream of RPA0928 was inserted into upstream flanking base 3696, downstream flanking base 3951 of pK18mobsacB. This deleted the whole coding sequence of RPA0928. | This study            |
| pBBR1MCS-5        | Broad host range cloning vector, Gm’ , lacZa, mob, mod, rep                         | Ref. 46                |

**Relevant genotype**: The table provides information about the relevant genotype of each strain or plasmid. This includes details about the specific deletion or insertion of genes or regulatory regions, which are crucial for understanding the biosynthetic capacity of methylated furan fatty acids.

**Source/Reference**: Each entry in the table is referenced to the appropriate literature, ensuring that readers can trace back to the original studies for further information. This allows for a comprehensive understanding of the genetic modifications and their impact on fatty acid biosynthesis.

**Plasmids**: The plasmids listed include pK18mobsacB, pRSBY1, and others, each with specific sequences and functions, essential for the study of methylated furan fatty acid biosynthesis and their role in R. palustris and R. sphaeroides.
nitrilotriacetic acid–agarose resin (Fisher/Pierce) for 30 min at 4 °C, poured into a column, and then washed with 50 ml of wash buffer I (50 mM Na2HPO4, pH 7.5, 1M NaCl, 35 mM imidazole) and 50 ml of wash buffer II (50 mM Na2HPO4, pH 7.5, 1M NaCl, 500 mM imidazole). Fractions containing the most protein were combined and concentrated in the wheat germ cell-free system.

In vitro assay of His8-UfaD activity

The phospholipid substrate was purified from a ΔufaD strain, and a phospholipid micelle solution (in water) was created (59) and quantitated by a lipid phosphorous assay (60). The enzyme reaction contained ~3 mM phospholipid and 0.7 μM His8-UfaD protein in 20 mM potassium phosphate buffer, pH 7.4, 0.5 mg/ml BSA and was incubated at 30 °C overnight.

Cell-free synthesis and in vitro assay of His8-UfaO and His8-FufM activity

pEU-ufaO was generated by cloning the ufaO coding region into pEU to produce an N-terminally hexahistidine-tagged protein (His6-ufaO and His6-FufM). Synthesis of both UfaO and FufM was achieved in a wheat germ cell-free extract using published translation conditions (61). The fatty acid modification reaction was run concurrently with the translation reaction by adding ~3 mM phospholipid substrate, prepared as described above, to the reaction. The phospholipid substrate was purified from a ΔufaO strain (for the 9M5-FuFA synthesis assay) and a ΔchrR strain (for the FuFA assay) to generate phospholipid lysosomes containing putative enzyme substrates and quantified as above (51). As determined by SDS-PAGE imaging with a Bio-Rad stain-free imaging system (61), the UfaO reaction contained ~60 μM UfaO; however, FufM concentration could not be calculated this way as it ran concurrently with other proteins in the wheat germ cell-free system.

18O2 modification of fatty acid chains

Cell-free protein synthesis was performed in the presence of 18O2 (99% isotopic enrichment, from Sigma–Aldrich, 602892-1L) in a sealed reaction tube prepared in an anaerobic hood or in air. Cultures were incubated in a sealed container, and a balloon with 18O2, N2, or compressed air was added to each culture. Data shown are representative results from duplicate technical replicates of each experiment.

Data availability

All data are contained within this article and the supporting information.

Acknowledgments—We thank Bryan Lakey for useful discussions and proofreading.

Author contributions—R. A. S. L., S. D. K., B. G. F., and T. J. D. conceptualization; R. A. S. L., S. D. K., A. H., E. T. B., J. R., and B. G. F. resources; R. A. S. L., K. M., S. D. K., and A. H. investigation; R. A. S. L., S. M. O., K. M., S. D. K., A. H., and E. T. B. methodology; R. A. S. L., K. M., S. D. K., A. H., and E. T. B. data curation; R. A. S. L., K. M., S. D. K., A. H., E. T. B., J. R., and B. G. F. writing-original draft; R. A. S. L., K. M., S. D. K., A. H., E. T. B., J. R., J. J. C., B. G. F., and T. J. D. writing-review and editing.

Funding and additional information—This work was supported in part by Department of Energy, Office of Science, Great Lakes Bioenergy Research Center Grant DE-SC0018409 (to T. J. D.).
Conflict of interest—The authors declare that they have no conflicts of interest with the contents of this article.

Abbreviations—The abbreviations used are: 9M5-FuFA, methyl 9-(3-methyl-5-pentylfuran-2-yl) nonanoate, or 10,13-epoxy-11-methyl-octadecadienoic acid; FuFA, furan fatty acid; FAME, fatty acid methyl ester; 11Me-12t-18:1, (11E)-methylloctadeca-12-enolic acid; 11Me-10t,12t-18:2, (10E,12E)-11-methylloctadeca-10,12-dienolic acid; 9D5-FuFA, methyl 9-(3,4-dimethyl-5-pentylfuran-2-yl) nonanoate; O₂, singlet oxygen; O₂, atmospheric oxygen; DMOX, 4,4-dimethyloxazoline; IPTG, isopropyl β-d-l-thiogalactoside.
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