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Solving a furan fatty acid biosynthesis puzzle

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Furan fatty acids (FuFAs), characterized by a central furan moiety, are widely dispersed in nature, but their biosynthetic origins are not clear. A new study from Lemke et al. employs a full court press of genetics, genomics, biochemical and advanced analytical techniques to dissect the biosynthetic pathway of mono- and di-methyl FuFAs and their intermediates in two related bacteria. These findings lay the foundation both for detailed study of these novel enzymes and for gaining further insights into FuFA function.

Furan fatty acids (FuFAs) are heterocyclic fatty acids produced in algae, plants and microorganisms, and taken up by animals in their diets (1,2). FuFAs are implicated as second messengers in cellular pathways that protect from oxidative damage (2), and their characteristic furan rings can also provide direct protection against oxidants by scavenging hydroxyl and peroxyl radicals (3). FuFAs have been shown to provide health benefits (4,5), and also have potential industrial uses as feedstocks for the production of lubricants, fuel additives, and biofuels (2). Understanding the full scope of FuFA biology and producing enough FuFAs for health and industrial applications would both benefit from having a defined biosynthetic pathway(s), but a complete pathway remains unknown.

The furans in FuFAs are typically found in the middle of the fatty acid chain, with a carboxyalkyl chain containing 7, 9, 11 or 13 carbon atoms and a propyl or pentyl side chain flanking each side; the furan rings can also be decorated with 0, 1 or 2 methyl groups (4). Fundamentally different fatty acid compositions in two algae species suggests that the pentyl- and propyl- side chains are derived from linoleic acid and 9,12-hexadecadienoic acid, respectively (Fig. 1) (6). The initial step involves a lipoxygenase-catalyzed oxidation to form 13-hydroperoxy-9,11-alkandienoic followed by electron shifts, ring closure and double bond rearrangement to produce the FuFA which becomes methylated (4). A distinct pathway was proposed in marine bacteria (7). Based on the intermediates that were isolated, FuFA biosynthesis was proposed to start with the methylation of cis vaccenic acid (also known as 18:1 11z) to form 11-Me-18:1, followed by desaturation to generate 11-Me-18:2, and finally incorporation of oxygen to form the furan ring (Fig. 1)(7). A similar pathway was also proposed in the photosynthetic bacterium *Rhodobacter sphaeroides* (3) after RSP2144 (UfaM) was identified as a SAM-dependent methylase that converts vaccenic acid to E-11-methyloctadeca-12-enoic acid (11Me-12t-18:1), a potential intermediate for FuFA biosynthesis. The previous report (4) also pointed to the RSP1087-1091 operon involved in FuFA synthesis, but the other intermediates and enzymes for subsequent reactions in this pathway remained unclear.

In the current study, Lemke et al. report elegant work defining the entire mono- and di-methyl FuFA biosynthesis pathway in photosynthetic bacteria (8). The authors had previously identified a *Rb. sphaeroides* mutant lacking the ChrR antisigma factor with increased abundance of FuFA, which served as a key to their success in dissecting the function of the RSP1087-1091 operon. The authors individually deleted each gene in the operon in the ΔChrR mutant, ruling out the involvement of RSP1087, 1088 and 1089. Deletion of RSP1091 led to depletion of methyl 9-(3-methyl-5-pentylfuran-2-yl) nonanoate (9M5-

FuFA) and accumulation of 11Me-12t-18:1, implying that RSP1091 plays an important role in producing an intermediate between 11Me-12t-18:1 and 9M5-FuFA. Deletion of RSP1090 in ΔChrR resulted in a new product that GC-MS and NMR analysis identified as the doubly unsaturated 11Me-10t,12t-18:2. The authors next generated a strain containing ChrR but lacking UfaM, RSP1091 and RSP1090, and found that the strain produced neither detectable FuFA nor its proposed intermediates. Overexpression of UfaM in this strain led to the accumulation of 11Me-12t-18:1, co-expression of UfaM and RSP1091 generated 11Me-10t,12t-18:2, and overexpression of all three genes led to the production of 9M5-FuFA. These results suggested that UfaM, RSP1091 and RSP 1090 might sequentially convert 11Me-12t-18:1 to 11Me-10t, 12t-18:2 and 9M5-FuFA, respectively (Fig. 1).

In vitro assays of recombinant protein incubated with their potential substrates confirmed the proposed reactions. RSP1091 (renamed UfaD) functions as a fatty acyl desaturase with homology to phytoene desaturase rather than other characterized fatty acid desaturases. UfaD's product was only observed when the cells were grown aerobically, suggesting the enzyme may use molecular oxygen in its catalytic reaction. Moreover, the mass of 9M5-FuFA generated in in vitro and cellular assays performed in the presence of an $^{18}O_2$ atmosphere increased by 2 Da compared to the 9M5-FuFA produced under an $^{16}O_2$ atmosphere, indicating that the O_2 is the source of the oxygen in the furan ring; these oxygen dependencies may help to explain a former report that FuFA only accumulated under aerobic conditions (3). Based on these results, the authors conclude that RSP1090 (renamed UfaO) functions as a fatty acid modifying enzyme that incorporates molecular oxygen into 11Me-10t, 12t-18:2 to produce 9M5-FuFA.

Genes related to UfaM, UfaD, and UfaO have been reported in other bacteria (9), begging the question as to whether or not they carry out the same biosynthetic functions. To answer this question, the authors studied the phostynthetic bacterium *Rhodopseudomonas palustris* homologs RPA2569, RPA2571 and RSA2570. Overexpression of these three homologs in the *Rb. sphaeroides* ΔUfaMΔUfaDΔUfaO triple mutant restored 9M5-FuFA production. Deleting the genes in the native strain abolished 9M5-FuFA accumulation, which could be rescued by introducing an appropriate plasmid. These data convincingly confirm the existence of a similar FuFA biosynthetic pathway in other bacteria.

Finally, in addition to 9M5-FuFA, the authors noted the presence in *Rs. palustrus* of the corresponding dimethyl compound, 9D5-FuFA. They tested the two genes (RPA3082 and RPA0924) annotated as SAM-dependent fatty acid modifying enzymes, and thus likely methylases, to see if either played a role in 9D5-FufA synthesis. While RPA3082 deletion had no effect on fatty acid profiles, inactivation of RPA0924 was associated with the loss of 9D5-FuFA consistent with a role in 9D5-FuFA synthesis, and its expression in *Rb. Sphaeroides* ΔChrR restored 9D5-FuFA accumulation. In vitro incubation of RPA0924 with SAM and 9M5-FuFA also resulted in 9D5-FuFA accumulation, confirming it as a 9M5-FuFA methylase (renamed FufM).

In conclusion, using a tour de force of biochemical-genetic approaches the authors identified and established the functions of three novel enzymes in the FuFAs biosynthetic pathway. Their work leaves some open questions, what's the function of adjacent RSP1087-1089? Are there any physiological benefits to further methylating 9M5-FuFA to 9D5-FuFA? Homologues of UfaM, UfaD and Ufa are found in other organisms, study of corresponding homologs may help identify important enzymes in FuFA biosynthesis in plants, algae and other organisms. Based on similarities in the pathways, could the RPA0924 homolog in algae work as a FufM (Fig. 1)? Fatty acyl monooxygenases are known to be involved in fatty acid metabolism (10), UfaO is a potential new class of monooxygenases, which catalyzes the ring formation to stabilize the chain to prevent cleavage. It is possible that this pathway is an evolutionary offshoot of fatty acid catabolism. The existence of distinct synthetic pathways in plants, algae and photosynthetic bacterium suggests the existence of a strong evolutionary pressure that resulted in convergent strategies to produce FuFA as radical scavengers for protection against deleterious effect of UV radiation. The present work(8) lays the foundation for further studies by creating and strains and

methodology to study enzymes and factors involved in FuFA biosynthesis and for engineering their accumulation as chemical feedstocks in plants and/or other production hosts.

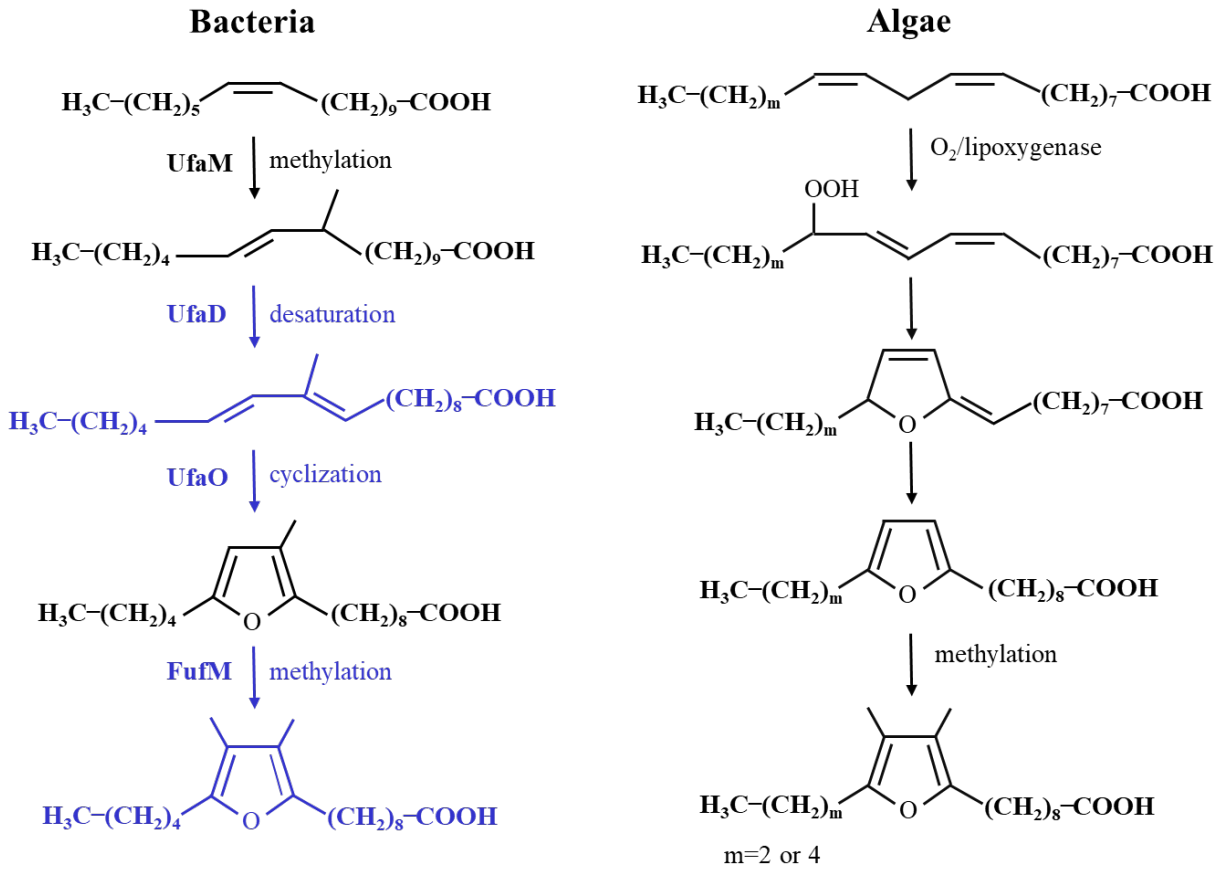

Figure 1. Biosynthesis of furan fatty acids. The biosynthesis pathway in bacterium (Left) (7,8) is contrasted with that proposed for algae (Right) (6). Newly identified enzymes and verified steps are colored in blue

The abbreviations used are: FuFA, furan fatty acids; 11Me-12t-18:1, E-11-methyloctadeca-12-enoic acid; 9M5-FuFA, methyl 9-(3-methyl-5-pentylfuran-2-yl) nonanoate.

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References

1. Xu, L., Sinclair, A. J., Faiza, M., Li, D., Han, X., Yin, H., and Wang, Y. (2017) Furan fatty acids - Beneficial or harmful to health? *Prog Lipid Res* **68**, 119-137
2. Spiteller, G. (2005) Furan fatty acids: occurrence, synthesis, and reactions. Are furan fatty acids responsible for the cardioprotective effects of a fish diet? *Lipids* **40**, 755-771

3. Lemke, R. A., Peterson, A. C., Ziegelhoffer, E. C., Westphall, M. S., Tjellstrom, H., Coon, J. J., and Donohue, T. J. (2014) Synthesis and scavenging role of furan fatty acids. *Proc Natl Acad Sci U S A* **111**, E3450-3457
4. Vetter, W., and Wendlinger, C. (2013) Furan fatty acids - valuable minor fatty acids in food. *Lipid Technology* **25**, 7-10
5. Li, K., Sinclair, A. J., Zhao, F., and Li, D. (2018) Uncommon Fatty Acids and Cardiometabolic Health. *Nutrients* **10**
6. Batna, A., Scheinkonig, J., and Spiteller, G. (1993) The occurrence of furan fatty acids in Isochrysis sp. and Phaeodactylum tricornutum. *Biochim Biophys Acta* **1166**, 171-176
7. Shirasaka, N., Nishi, K., and Shimizu, S. (1997) Biosynthesis of furan fatty acids (F-acids) by a marine bacterium, Shewanella putrefaciens. *Biochim Biophys Acta* **1346**, 253-260
8. Lemke, R. A. S., Olson, S. M., Morse, K., Karlen, S. D., Higbee, A., Beebe, E. T., Ralph, J., Coon, J. J., Fox, B. G., and Donohue, T. J. (2020) A bacterial biosynthetic pathway for methylated furan fatty acids. *J Biol Chem*
9. Dufour, Y. S., Landick, R., and Donohue, T. J. (2008) Organization and evolution of the biological response to singlet oxygen stress. *J Mol Biol* **383**, 713-730
10. Van Bogaert, I. N., Groeneboer, S., Saerens, K., and Soetaert, W. (2011) The role of cytochrome P450 monooxygenases in microbial fatty acid metabolism. *FEBS J* **278**, 206-221