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Biosynthesis of fatty acid-derived hydrocarbons: perspectives on enzymology and enzyme engineering

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Enormous fuel consumption and growing environmental concerns have been spurring development of renewable biofuels. Among various biofuels, fatty acid-derived biohydrocarbons are ideal alternatives to non-renewable fossil fuels due to their closest properties to petroleum-based fuels. In the past decade, novel hydrocarbon-producing enzymes have continuously been discovered and engineered. Here, we review the recent advances in biosynthesis of fatty acid-derived hydrocarbons with emphasis on enzymology and enzyme engineering, based on which some outlooks are provided.

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Introduction

The ever-growing demand for energy and excessive reliance on fossil fuels have led to a severe environmental and global climate crisis with many adverse impacts on human society and economy [1]. Driven by depletion of non-renewable fossil fuels and awakening public environmental concerns, biofuels such as bioethanol, biodiesel and biohydrocarbons from renewable resources have been considered as sustainable substitutes for traditional fuels [2]. Each biofuel class has certain societal and technological trade-offs. For instance, bioethanol production is technically mature, but the relatively lower energy content and hygroscopicity of bioethanol limits its application scope while edible oil-derived biodiesel competes against the use of these oils in the food sector. Biohydrocarbons (alkanes and alkenes) possess high energy contents and are more compatible with petroleum-derived fuels, but require more research investment and development to deploy in commercial production. Although biohydrocarbons can be derived from several metabolic pathways, this review is focused on fatty acid-derived biohydrocarbons (hereinafter referred to as biohydrocarbons unless otherwise specified). For terpene hydrocarbons and gaseous biohydrocarbons methane and ethylene, some excellent reviews can be found elsewhere [3••,4–6].

Biohydrocarbons can be synthesized by microbes (bacteria, cyanobacteria and fungi) [7], insects [8], plants [9], and microalgae [10]. Their known physiological functions include protection from ambient stresses, restriction of surface water loss, storage of intermediates, and intercellular communications. However, the natural biohydrocarbon yields are generally low, which prevents their direct utilization as biofuels. Recent enthusiasm for biosynthesis of hydrocarbons has been inspired by the discovery of an alkane biosynthetic pathway from the cyanobacterium *Synechococcus elongatus* PCC 7942 [11]. Thus far, three types of biohydrocarbons stemming from fatty acid biosynthetic pathway have been discovered, namely alkanes, terminal alkenes (i.e. 1-alkenes) and internal alkenes, all of which are ideal alternatives to fossil fuels.

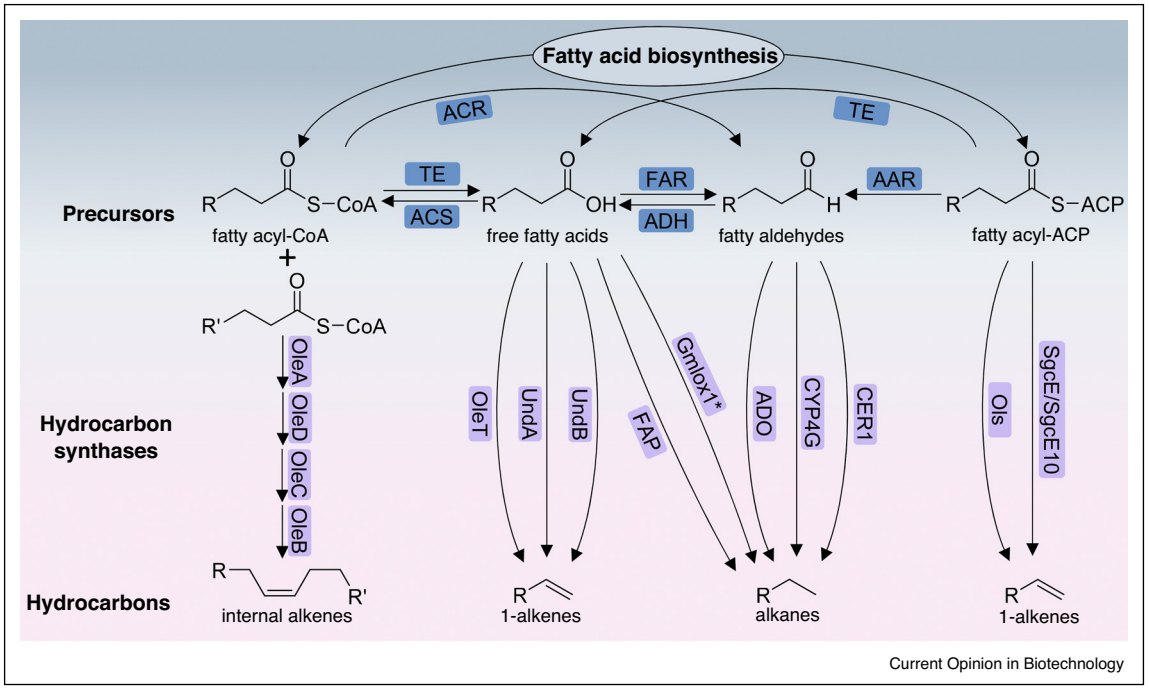
Although hydrocarbon biosynthesis has been reviewed from different angles [3••,12,13], the purpose of this review is to update the most recent advances in this fast-progressing field, with emphasis on enzymology (Figure 1, Table 1) and enzyme engineering (Figure 2) for the biocatalysts directly responsible for hydrocarbon production. The following sections are organized according to the types of fatty acyl substrates for all characterized hydrocarbon-producing enzymes so far, including free fatty acids (FFAs), fatty aldehydes (FAHs), fatty acyl–acyl carrier proteins (acyl–ACPs), and fatty acyl coenzyme A (acyl–CoAs).

Enzymes converting FFAs to 1-alkenes or alkanes

OleT

1-Alkenes are key commodity chemicals and petrochemical intermediates used not only as fuel quality additives, but also for making lubricants, polymers and detergents. The cytochrome P450 peroxygenase $OleT_{JE}$, first identified from *Jeotgalicoccus* sp. ATCC 8456, catalyzes the oxidative decarboxylation of long-chain (C_{16}–C_{20}) fatty acids to produce C_{n-1} 1-alkenes, with H_2O_2 as cofactor and CO_2 as co-product. $OleT_{JE}$ also generates α-hydroxyl

Figure 1



Biosynthetic pathways for fatty acid-derived hydrocarbons. Precursor-generating and hydrocarbon-producing enzymes are shown in blue and purple boxes, respectively. Abbreviations: ACR, acyl–CoA reductase; TE, thioesterase; FAR: fatty acid reductase; AAR, acyl–ACP reductase; ACS, acyl–CoA synthetase; ADH, aldehyde dehydrogenase. *See text for details.

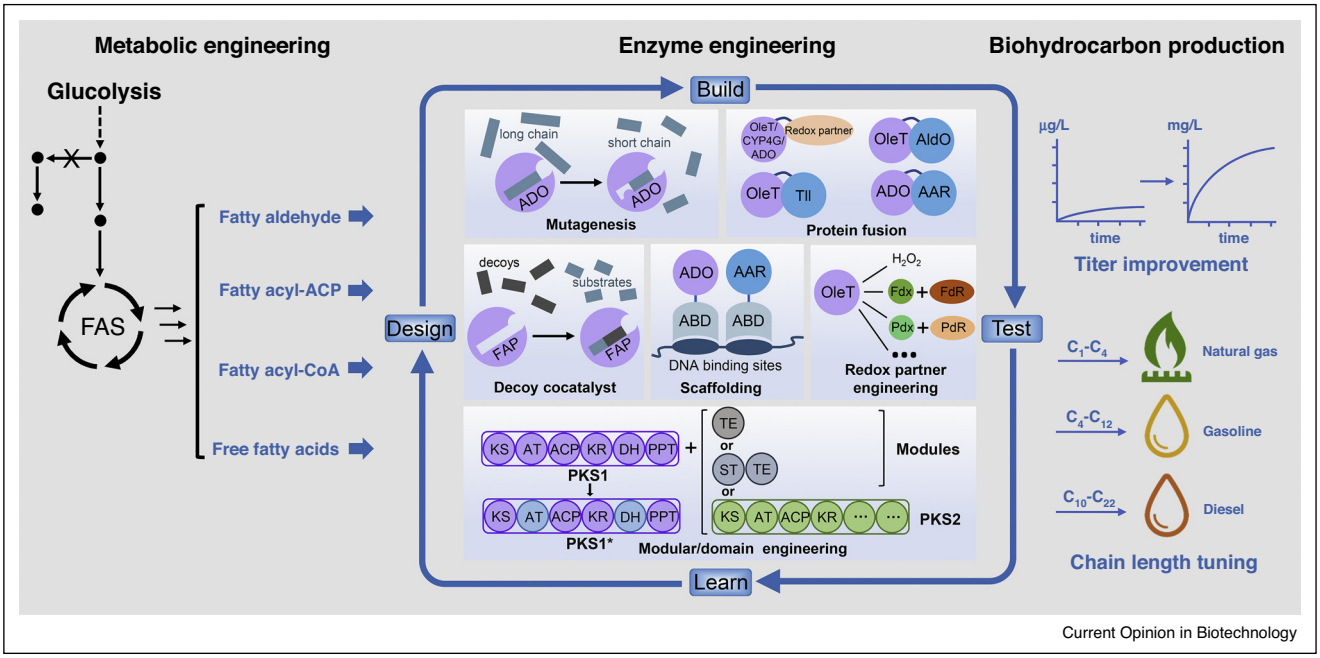
Table 1

Biosynthetic enzymes for fatty acid-derived hydrocarbons

Substrate	Enzyme/characteristics	Substrate chain length/ product/byproduct	Cofactors	Source	Year	First reference
Free fatty acid	OleT/P450 decarboxylase	C_4–C_{20}/1-alkenes (C_{n-1})/CO_2	Heme, H_2O_2 or NADPH/redox partners	Bacteria	2011	[14]
	UndA/non-heme iron decarboxylase	C_{10}–C_{14}/1-alkenes (C_{n-1})/CO_2	Fe^{2+}	Bacteria	2014	[28]
	UndB/membrane-bound desaturase-like decarboxylase	C_6–C_{18}/1-alkenes (C_{n-1})/CO_2	Fe^{2+}	Bacteria	2015	[30]
	FAP/fatty acid photodecarboxylase	C_1–C_{18}/alkanes (C_{n-1})/CO_2	FAD	Microalgae	2017	[32••]
	Gmlox1/Lipoxygenase	Linoleic acid (C_{18})/pentane (C_5)/ 13-oxo-*cis*-9-*trans*-11-tridecadienoic acid	Fe^{2+}	Soybean	2013	[34]
Fatty aldehyde	ADO/aldehyde deformylating oxygenase	C_4–C_{18}/alkanes (C_{n-1})/formate	Fe^{2+}, NADPH/redox partners	Cyanobacteria	2010	[11]
	CYP4G/P450 aldehyde decarbonylase	C_{24}–C_{28}/alkanes (C_{n-1})/CO_2	Heme, NADPH, CPR	Insects	2012	[45]
	CER1/aldehyde decarbonylase	C_8–C_{16}, C_{28}–C_{34}/alkanes (C_{n-1})/CO	Fe^{2+}, CYTB5	Plants	2012	[44]
Fatty acyl–ACP	Ols/type-I PKS	C_{17}–C_{18}/1-alkenes (C_{n+1})/CO_2	NADPH, PAPS	Cyanobacteria	2011	[49]
	SgcE, SgcE10/iterative PKS	C_3/pentadecaheptaene (C_{15})/CO_2	NADPH	Bacteria	2015	[54]
Fatty acyl–CoA	OleABCD/α/β-hydrolase[a]	C_{10}–C_{15}/internal alkene ($C_{n+n'-1}$)/CO_2	–	Bacteria	2010	[57]

[a] OleB directly responsible for production of *cis*-alkenes is an α/β-hydrolase.

Figure 2



Schematic diagram of enzyme engineering work described in this review. Abbreviations: FAS, fatty acid synthesis; ABD, artificial DNA-binding domain.

and β-hydroxyl fatty acids as side products [14]. Afterwards, more OleT enzymes have been bioinformatically discovered and biochemically characterized [15•,16], some of which show moderate halophilic properties, thus holding the potential for 1-alkene production either *in vitro* or *in vivo* under high salinity conditions (e.g. in seawater).

It has been accepted that the $OleT_{JE}$ catalyzed reaction is initiated by the formation of an iron(IV)-oxo cation radical (Compound I) upon heterolytic cleavage of the O–O bond in H_2O_2. The canonical hydrogen atom abstraction by Compound I generates a substrate radical at the C_β position. The decarboxylation/hydroxylation bifurcation results from either an additional C_β electron abstraction to form a substrate carbocation for decarboxylation or normal oxygen rebound for hydroxylation [17,18]. Based on this mechanism and the first crystal structures of $OleT_{JE}$ [19], a great deal of enzyme engineering efforts have been made for improved decarboxylation activity. However, almost all attempts on $OleT_{JE}$ at the active-site residues have only led to neutral or even compromised decarboxylation activity [20,21], highlighting the subtle control of $OleT_{JE}$ catalytic behaviors and the difficulty to (semi-) rationally design a better decarboxylase. Thus, the first and prototypic $OleT_{JE}$ remains the most efficient P450 FFA decarboxylase among all characterized OleT enzymes.

Interestingly, various heterologous redox partner proteins, including the fused P450 reductase domain RhFRED and separate flavodoxin/flavodoxin reductase [22], and a total of 30 combinations of ferredoxin/ferredoxin reductase (Fdx/FdR) from (cyano)bacteria [20], were found to be able to alternatively support the $OleT_{JE}$ activity. Moreover, an optimization of the $OleT_{JE}$/redox partners/NAD(P)H reaction system using putidaredoxin/putidaredoxin reductase (Pdx/PdR) as surrogate redox partners and formate dehydrogenase or phosphite dehydrogenase for NAD(P)H regeneration, achieved the product titers of C_3–C_8 alkenes up to 0.93 g/L and total turnover numbers >2000 [23]. Thus, using redox partners to drive OleT catalysis has been proven as an effective method for construction of efficient biocatalytic systems for 1-alkene production, even though it was later determined that the supportive function of redox partners is actually owing to H_2O_2 derived from the decoupled electron transfer [15•,24•]. The first $OleT_{JE}$–RhFRED fusion protein that successfully converted lauric acid into 1-undecene [22] has inspired more $OleT_{JE}$ fusion proteins. For example, the fusion of $OleT_{JE}$ with the reductase domain of a self-sufficient P450 BM3 showed enhanced decarboxylation and broad substrate scope [25]. Apart from redox partners, AldO, an alditol oxidase that oxidizes glycerol with H_2O_2 as co-product, was fused to $OleT_{JE}$ to supply H_2O_2 *in situ*. Upon this fusion, addition of 1% glycerol gave an 8% increase in myristic

acid conversion compared to the addition of 0.5 mM H_2O_2 [26]. Furthermore, a lipase Tll was fused to $OleT_{JE}$, which enables the chimeric enzymes to transform triglycerides into 1-alkenes [27].

UndA and UndB

Inspired by the phenomenon that the semi-volatile 1-undecene is ubiquitously produced by *Pseudomonas* spp., Rui *et al.* identified the responsible gene product UndA from various *Pseudomonas* species by gene disruption and heterologous expression in *Escherichia coli* [28]. Unlike OleT hemoproteins, UndA is a nonheme iron(II)-dependent oxidase catalyzing the decarboxylation of medium-chain FFAs (C_{10}–C_{14}) to C_{n-1} 1-alkenes, whose mechanism was recently elucidated [29•]. Later, the same group discovered a new family of membrane-bound fatty acid desaturase-like enzymes, named UndB, that convert C_6–C_{18} FFAs into C_{n-1} 1-alkenes through oxidative decarboxylation [30]. However, UndB homologues are not as widespread as UndA family members. Mechanistically, OleT, UndA, and UndB all initiate their catalysis via β-hydrogen abstraction, demonstrating an interesting event of convergent evolution following a reasonable chemical logic. Despite the membrane-bound property, UndB appeared to be more efficient in producing 1-alkenes (especially 1-undecene) *in vivo* than $OleT_{JE}$ and UndA in both *E. coli* [30] and *Sacchromyces cerevisiae* [31•]. One possible reason is that UndB might facilitate hydrocarbon export [31•].

FAP

From the algal hydrocarbon-producer *Chlorella variabilis* NC64A, a fatty acid photodecarboxylase (FAP), which belongs to the glucose-methanol-choline oxidoreductase family, was shown to catalyze the decarboxylation of FFAs (C_{12}–C_{22} with C_{16}–C_{17} preferred) to form C_{n-1} alkanes [32••]. The FAP-mediated decarboxylation is initiated via electron abstraction from the FFA substrates by the photo-excited flavin adenine dinucleotide (FAD) with a quantum yield >80%, which requires a constant input of blue photons to drive the catalytic cycle. The resultant FFA radical decarboxylates to an alkyl radical and eventually release the alkane product after a cascade of electron transfers. Recently, a range of short-chain carboxylic acids (C_1–C_6) were demonstrated to be converted into H_2 or C_{n-1} alkanes by using simple alkanes (C_7–C_{17}) as decoy molecules to fill up the vacant substrate channel of FAP [33•]. This approach exhibits a novel strategy for short-chain alkane production.

Gmlox1

Unlike the above decarboxylases, the soybean lipoxygenase I (Gmlox1) uses a distinct strategy to make alkanes. Specifically, Gmlox1 catalyzes the insertion of O_2 into a *cis* double bond of linoleic acid (C18:2) to form 13-hydroperoxylinoleic acid (13-HPOD). The following homolytic-β-scission converts 13-HPOD into pentane and the byproduct 13-oxo-*cis*-9-*trans*-11-tridecadienoic acid. Through heterologous overexpression of Gmlox1 in the oleaginous yeast *Yarrowia lipolytica*, together with medium optimization and metabolic engineering, a titer of 5 mg/L pentane was achieved [34]. Despite the abundance of linoleic acid in nature and the uniqueness of this internal double bond cleavage, the equal molar production of a large byproduct may hinder its further commercial development unless large-volume applications for the byproduct are found.

Enzymes converting FAHs to alkanes

ADO

The discovery of the alkane biosynthetic pathway consisting of an acyl–ACP reductase (AAR) and an aldehyde deformylating oxygenase (ADO) was a prelude of the global rush for hydrocarbon-producing enzymes [11]. The precursor FAHs are generated from the reduction of acyl–ACPs by AAR. Subsequently, the non-heme di-iron enzyme ADO transforms FAHs (C_{14}–C_{18}) to corresponding C_{n-1} alkanes with formate as byproduct [35]. The heterologous expression of AAR/ADO in *E. coli* led to 0.3 g/L C_{13}–C_{17} alkane mixtures [11]. AAR/ADO were also functionally expressed in the filamentous fungus *Aspergillus carbonarius*, producing 0.2–10.2 mg/L C_{15}–C_{17} alkanes in different media [36].

On the basis of structural analyses of ADOs [37,38], a group of residues that constitute the substrate binding tunnel were identified and rationally mutated, and the substrate selectivity was successfully changed to prefer shorter-chain aldehydes [37,39]. A significant problem for ADO-mediated alkane synthesis is the endogenous substrate competition by aldehyde reductases and alcohol dehydrogenases [40]. To circumvent this problem, in addition to targeted deletion of competing enzymes [40,41], both a chimeric ADO–AAR fusion protein and a zinc-finger protein-guided ADO/AAR assembly on DNA scaffolds were constructed to establish substrate channeling effects, by which 4.8-fold and 8.8-fold increases in alkane production by engineered *E. coli* strains were achieved, respectively [42]. Moreover, since ADO requires redox partners to provide electrons for catalysis, some ADO-redox partner fusions were also made for efficiency improvement [43].

CYP4G and CER1

In addition to prokaryotic ADOs, two classes of eukaryotic aldehyde decarbonylases can also convert long-chain FAH into C_{n-1} alkanes, including the cytochrome P450 decarbonylases (CYP4G) from insects and the endoplasmic reticulum (ER) membrane-bound protein ECERIFERUM1 (CER1) from plants. Notably, CYP4G and CER1 generate CO_2 and CO as respective co-product [44,45], indicating their distinct catalytic mechanisms.

Heterologous expression of *Drosophila* CYP4G1 and its cognate redox partner protein NADPH–cytochrome P450 reductase (CPR) in *S. cerevisiae* resulted in production of $C_{23–27}$ alkanes from $C_{24–28}$ aldehydes. Moreover, the house fly CYP4G2–CPR fusion protein was expressed in *Sf9* insect cells, and the recovered microsomes successfully converted octadecanal to heptadecane [45]. Recently, CYP4G55 and CYP4G56 from mountain pine beetle *Dendroctonus ponderosae* were found to produce alkanes from both FAHs and fatty alcohols, raising some mechanistic questions for these P450 decarbonylases [46].

CER1 was proposed to be a di-iron containing aldehyde decarbonylase, which is an essential element for wax alkane synthesis in *Arabidopsis* [47]. Further screening for CER1 physiological partners revealed that CER1 interacts with the wax-associated protein ECERIFERUM3 (CER3) and an ER–associated cytochrome b5 (CYTB5) isoform to supply electrons. Reconstitution of CER1/CER3 mediated alkane production was achieved in *S. cerevisiae*, leading to very long chain alkane synthesis, which was further enhanced by addition of CYTB5 [44]. Intriguingly, expression of CER1 in *E. coli* resulted in production of short-chain alkanes, the major components of gasoline [48]. However, the paucity of mechanistic understanding of CER1/CER3/CYTB5 is a barrier to further optimization of this alkane-producing system.

Enzymes converting acyl–ACPs to 1-alkenes

Ols

Acyl–ACPs are common substrates for fatty acid synthases (FASs) and polyketide synthases (PKSs). Mendez-Perez *et al.* first discovered Ols, a multi-domain type-I PKS from cyanobacterium *Synechococcus* sp. PCC 7002 that is able to synthesize C_{n+1} 1-alkenes from C_n acyl–ACPs via an elongation/sulfonation/decarboxylation process [49]. First, the loading domain (LD) uploads a C_n FFA substrate onto the ACP1 domain. Next, the ketosynthase (KS), acyltransferase (AT), ketoreductase (KR) and ACP2 domains in the central extension module act sequentially to generate the β-hydroxyl C_{n+2} acyl–ACP. Finally, a unique 3′-phosphate-5′-phosphosulfate (PAPS)-dependent sulfotransferase (ST) domain and a thioesterase (TE) domain co-mediate a sulfate elimination assisted decarboxylative dehydration of the C_{n+2} acyl–ACP substrate, giving rise to the final C_{n+1} 1-alkene product. Interestingly, this strategy for terminal olefin generation is also employed by the curacin biosynthetic pathway in *Lyngbya majuscule* [50] and many other cyanobacteria [51]. Heterologous expression of various natural and hybrid ST-TE-containing PKSs has enabled production of different 1-alkenes such as propene, butadiene and 1-hexene in *E. coli* [52].

SgcE/SgcE10

FASs and PKSs utilize common chemistry with the latter being able to control the chain length of products in a more accurate and controllable manner. Thus, PKSs hold great potential to be engineered for producing single-component hydrocarbons rather than most reported alka(e)ne mixtures. For instance, an iterative PKS SgcE and its cognate TE SgcE10 are key to biogenesis of the enediyne core structure in a number of antitumor natural products. Heterologous expression of SgcE/SgcE10 led to significant accumulation of the intermediate pentadecaheptaene [53]. Through fine-tuning expression of SgcE/SgcE10 in *E. coli*, a fed-batch fermentation of the engineered strain followed by flow chemical hydrogenation produced approximately 140 mg/L of pentadecane as the sole hydrocarbon product [54].

Collectively, the diversity and controllability of PKSs make these modular megasynthases promising enzymes for efficient production of different hydrocarbons. Particularly, the TE domains with decarboxylation functionalities, including the studied ST-TE, SgcE10, and CalE7 [55] and the unexplored tautomycetin TE and *Botryococcus brauii* hydrocarbon-producing TE [56], may be worth special attention because their abilities to generate terminal double bond could be linked to the engineered biocatalytic systems that can produce appropriate β-hydroxylated acyl–ACP precursors.

Enzymes converting acyl–CoAs to internal alkenes

The four genes *oleABCD* distributed in various phyla of bacteria were demonstrated to catalyze the unique head-to-head nondecarboxylative thiolytic condensation of two acyl–CoAs for long-chain internal alkene production [57,58]. The genes are located either tandemly or separately in genomes with *oleCD* always clustered even when *oleBC* are fused as a single gene [59]. Functionally, OleA catalyzes the non-decarboxylative Claisen condensation of two long-chain fatty acyl–CoAs to form a β-ketoacid and release two CoAs [60,61]. OleD, an NADPH-dependent reductase, stereospecifically reduces the β-ketoacid to a β-hydroxyl carboxylic acid [62], which is then converted to a β-lactone by OleC [63]. Finally, the α/β-hydrolase OleB catalyzes the decarboxylation of *cis*-β-lactones to yield *cis*-alkene products [64•]. Together, the four-step pathway transforms two acyl–CoAs ($C_{10–15}$ + $C_{10–15}$) into a $C_{19–29}$ *cis*-alkene, which is within the carbon range of diesel, heavy fuel oil, or wax.

Conclusions and perspectives

Enzymatic FFA deoxygenation can be achieved through deformylation, decarbonylation, and decarboxylation. All known hydrocarbon-producing enzymes (Table 1) adopt three distinct catalytic strategies: 1) For unactivated FFA and FAH substrates, all oxidative hydrocarbon synthases (i.e. OleT, UndA, UndB, Gmlox1, ADO, CYP4G, and

CER1) except FAP take advantage of different iron species to overcome the high energy barriers of deoxygenation reactions; 2) FAP uses the photo-excited FAD to decarboxylate FFA via a monoradical mechanism; 3) For β-activated intermediates derived from fatty acyl–CoA/ACP substrates, non-oxidative-coupled decarboxylation/elimination is utilized for alkene generation by OleB, Ols, and SgcE10.

Discovering more fatty acid deoxygenases with novel mechanisms, altered substrate specificities, and higher catalytic efficiencies apparently represents a frontier of this field. However, current biohydrocarbon titers are still cost-prohibitive and must be addressed through enzyme engineering, metabolic engineering, and process engineering to achieve commercial relevance. Considering that the highest FFA titers have exceeded 10 g/L [65] and the FFA chain length can be controlled by manipulating FAS/PKS modules or by expressing heterologous chain-specific thioesterases [66–68], the present hydrocarbon cell factories with low yields (in mg/L scale) apparently have major challenges to overcome such as low enzyme activities, problematic overexpression, insufficient cofactor supply, inefficient electron transfer by suboptimal redox partners, and oxidative stress caused by uncoupled electrons.

To address these challenges, significant enzyme engineering efforts including random/rational mutagenesis [15•,37,39], redox partner engineering [20,22,23], protein fusion [22,26,27,42,43,45] and scaffolding [42], modular/domain engineering [52,54] and the application of decoy molecules [33•] have been made. Furthermore, synthetic biology will play important roles in this frontier field. For example, 1) modular engineering of a self-balanced hydrocarbon metabolic network would significantly improve the total titers; 2) Engineering a non-natural dehydratase via *de novo* design and/or directed evolution that can act on the high-titer but unexploited fatty alcohol substrates may lead to a novel efficient hydrocarbon biosynthetic pathway branched from fatty alcohols; and 3) engineered compartmentalization of hydrocarbon biosynthetic steps could avoid the toxicity resulted from substrates/products, enzymes and cofactors [69]. Given the fast-emerging novel concepts, methods and technologies, we look forward to seeing industrial-scale production of biohydrocarbons in the foreseeable future.

Conflict of interest statement

Nothing declared.

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