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Hydrogen peroxide-independent production of α -alkenes by OleT_{JE} P450 fatty acid decarboxylase

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Abstract

Background: Cytochrome P450 OleT_{JE} from *Jeotgalicoccus* sp. ATCC 8456, a new member of the CYP152 peroxygenase family, was recently found to catalyze the unusual decarboxylation of long-chain fatty acids to form α -alkenes using H₂O₂ as the sole electron and oxygen donor. Because aliphatic α -alkenes are important chemicals that can be used as biofuels to replace fossil fuels, or for making lubricants, polymers and detergents, studies on OleT_{JE} fatty acid decarboxylase are significant and may lead to commercial production of biogenic α -alkenes in the future, which are renewable and more environmentally friendly than petroleum-derived equivalents.

Results: We report the H₂O₂-independent activity of OleT_{JE} for the first time. In the presence of NADPH and O₂, this P450 enzyme efficiently decarboxylates long-chain fatty acids (C₁₂ to C₂₀) *in vitro* when partnering with either the fused P450 reductase domain RhFRED from *Rhodococcus* sp. or the separate flavodoxin/flavodoxin reductase from *Escherichia coli*. *In vivo*, expression of OleT_{JE} or OleT_{JE}-RhFRED in different *E. coli* strains overproducing free fatty acids resulted in production of variant levels of multiple α -alkenes, with a highest total hydrocarbon titer of 97.6 mg·l⁻¹.

Conclusions: The discovery of the H₂O₂-independent activity of OleT_{JE} not only raises a number of fundamental questions on the monooxygenase-like mechanism of this peroxygenase, but also will direct the future metabolic engineering work toward improvement of O₂/redox partner(s)/NADPH for overproduction of α -alkenes by OleT_{JE}.

Keywords: Alkenes, Biofuels, Monooxygenase, P450 fatty acid decarboxylase, Peroxygenase

Background

The urgency to develop sustainable fossil fuel alternatives is driven by rapidly increasing global consumption, irreversibly diminishing reserves, unpredictable geopolitical factors, fluctuating price of crude oil, growing concerns about national energy security, and serious environmental concerns surrounding immense greenhouse gas emissions mainly resulting from combustion of fossil fuels [1-3]. Biofuels produced from biological resources represent a compelling alternative to fossil fuels because they are renewable and more environmentally friendly [4-7]. Among various biofuels, bioethanol and biodiesel are dominating the current global market. However, it is widely accepted that the ideal biofuels are biohydrocarbons, especially the medium- to long-chain fatty

alkanes or alkenes, because they highly mimic the chemical composition and physical characteristics of petroleum-based fuels [8-10]. Thus, the biosynthetic pathways for aliphatic hydrocarbons from diverse organisms have been attracting great attentions in recent years [11-13]. Particularly, the scalable and cost-effective microbial biosynthesis of fatty alkanes or alkenes is considered as one of the most promising ways to produce 'drop-in compatible' biofuels [5,8].

To date, four microbial biosynthetic pathways that convert free fatty acids or fatty acid thioesters into biohydrocarbons have been identified, including the cyanobacterial pathways consisting of an acyl-acyl carrier protein (acyl-ACP) reductase and an aldehyde decarboxylase, which together convert fatty acyl-ACPs into alkanes [14]; a three-gene cluster responsible for generating alkenes with internal double bonds through the head-to-head condensation of two fatty acyl-coenzyme A (acyl-CoA) molecules in *Micrococcus luteus* [15]; a unique P450 decarboxylase OleT_{JE} from *Jeotgalicoccus* sp. ATCC 8456, which directly decarboxylates long-

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chain fatty acids to form α -olefins in presence of H_2O_2 [16,17]; and a type I polyketide synthase from *Synechococcus* sp. PCC 7002 [18,19], which is capable of transforming fatty acyl-ACPs into α -olefins via sequential polyketide synthase chain elongation, keto reduction, sulfonation mediated by its sulfotransferase domain, and the coupled hydrolysis and decarboxylation catalyzed by the thioesterase domain.

Among these pathways, the single-step decarboxylation of fatty acids catalyzed by OleT_{JE} P450 enzyme [16] apparently represents the simplest one. Moreover, it directly uses free fatty acids instead of fatty acid thioesters as substrates, which is believed to be advantageous for metabolic engineering because fatty acids are more abundant and their abundance and composition can be well manipulated in *E. coli* [20-22], one of the most developed microbial cell factories. Thus, this P450 fatty acid decarboxylative machinery may hold great potential to be engineered into a biological α -alkene-producing system. Notably, in addition to being biofuel molecules, α -alkenes are also used broadly for making lubricants, polymers and detergents [17,23,24].

The superfamily of cytochrome P450 enzymes (CYPs) are considered among the most versatile biocatalysts in nature [25]. Typically, P450 enzymes employ one or more redox partner proteins to transfer two electrons from NAD(P)H to the heme iron reactive center for dioxygen activation, and then insert one atom of O_2 into their substrates [25-27]. Therefore, these oxidative biocatalysts are often termed as P450 monooxygenases. However, the CYP152 family members such as P450_{SP α} [28], P450_{BS β} [29] and OleT_{JE} [16] have been identified to exclusively use H_2O_2 as the sole electron and oxygen donor, and are thus classified into a unique P450 peroxygenase category.

Practically, the peroxygenase activity of P450 enzymes is often treated as an advantageous feature because H_2O_2 is much cheaper than NADPH and redox proteins in many P450 applications such as biocatalysts for *in vitro* synthetic reactions and enzyme additives in laundry detergents [30,31]. However, the large-scale production of low-cost α -alkene biofuels by OleT_{JE} P450 decarboxylase cannot rely on the H_2O_2 -dependent enzymatic system because the use of large amounts of peroxide is cost prohibitive, and high concentration of H_2O_2 can quickly deactivate biocatalysts [32].

Therefore, the H_2O_2 -independent activity of OleT_{JE}, if it exists, is preferred for cost-effective microbial production of α -alkenes. In the present study, we demonstrated such activity for the first time. Through engineering a self-sufficient version of OleT_{JE} by fusing it to the *Rhodococcus* fusion reductase (RhFRED) domain from *Rhodococcus* sp. NCIMB 9784 [33], the catalytic activity of the resultant fusion P450 enzyme can be solely driven by

NADPH. We also found that *E. coli* flavodoxin (Fld) and flavodoxin reductase (Fdr) are capable of supporting OleT_{JE} activity as well. Guided by these new findings, our initial metabolic engineering efforts based on the H_2O_2 -independent decarboxylation of fatty acids by OleT_{JE} *in vivo* led to a group of α -alkene overproducers with the best one producing 97.6 mg·l⁻¹ total α -alkenes.

Results

In vitro fatty acid decarboxylation by OleT_{JE} and OleT_{JE}-RhFRED

The unusual activity of the first P450 fatty acid decarboxylase OleT_{JE} was determined by reconstituted *in vitro* reactions [16]. Specifically, OleT_{JE} was able to decarboxylate stearic acid and palmitic acid to generate 1-heptadecene and 1-pentadecene, respectively, in the presence of H_2O_2 (Figure 1A). It also catalyzed the α - and β -hydroxylation of fatty acids as side reactions. Using the purified P450 enzymes (Additional file 1: Figure S1 and Additional file 2: Figure S2), we first confirmed these three types of activity of OleT_{JE} using myristic acid (C₁₄) as substrate. Consistent with the previous report [16], fatty acid decarboxylation was the dominant reaction, while the α - and β -hydroxymyristic acid only accounted for 0.2% and 6.1% of total products, respectively (Figure 1B).

According to previous amino acid sequence analysis, OleT_{JE} was assigned to the CYP152 family, with the well-studied P450_{BS β} [29,34,35] from *Bacillus subtilis* and P450_{SP α} [28,36] from *Sphingomonas paucimobilis* as family members. Essentially, this family of P450 enzymes utilizes H_2O_2 as the sole electron and oxygen donor to oxidize their substrates, in contrast to most other P450 monooxygenases, whose catalytic activity depends on dioxygen, NAD(P)H and redox partner protein(s) [37]. Thus, these P450 enzymes are functionally referred to as peroxygenases [38]. Indeed, it was reported that P450 reductase systems such as ferredoxin and ferredoxin reductase did not support the activity of P450_{BS β} and P450_{SP α} [36,39]. This is consistent with the substitution of Pro²⁴⁶ and Arg²⁴⁵ (numbering in OleT_{JE}) in CYP152 peroxygenases for the highly conserved threonine and an acidic residue (for example, Glu or Asp) in most P450 monooxygenases (Additional file 3: Figure S3), which are involved in a specific H-bond network responsible for delivery of protons during the P450 catalytic cycle [34,40].

However, in the study of another CYP152-member P450_{CLA}, from the anaerobic microorganism *Clostridium acetobutylicum* [41], it was demonstrated that both P450_{CLA} and P450_{BS β} functioned when provided with O_2 , NADPH and flavodoxin/flavodoxin reductase from *E. coli* or the diflavin reductase domain of P450_{BM3} from *Bacillus megaterium*, suggesting an alternative monooxygenase-like mechanism of these P450 peroxygenases.

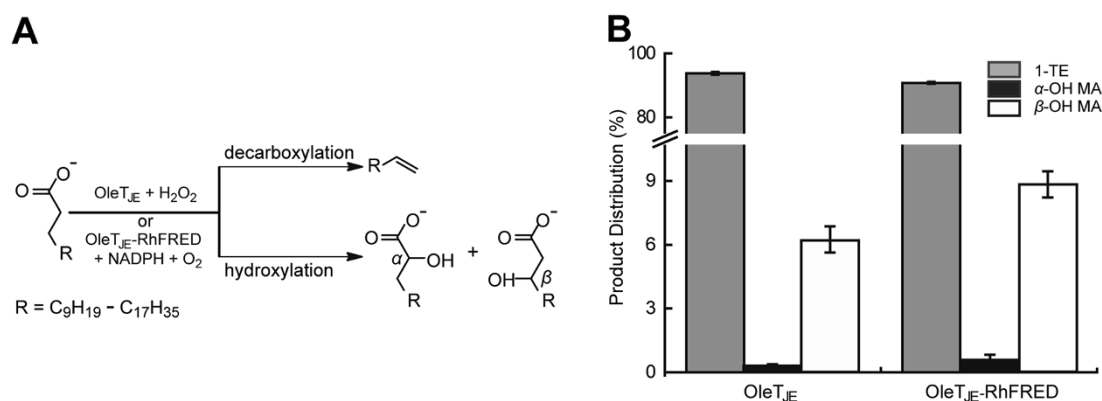


Figure 1 Decarboxylation and hydroxylation reactions catalyzed by OleT_{JE}. (A) The decarboxylation and hydroxylation of fatty acids (C₁₂ to C₂₀) catalyzed by OleT_{JE}. (B) Product distribution of the decarboxylation and hydroxylation reactions catalyzed by purified OleT_{JE} and OleT_{JE}-RhFRED, respectively. Myristic acid was used as substrate. 1-TE, 1-tridecene; α -OH MA, α -hydroxy myristic acid; β -OH MA, β -hydroxy myristic acid.

To investigate whether OleT_{JE} peroxygenase can also function as a monooxygenase like P450_{CLA} and P450_{BSB}, the RhFRED reductase domain from *Rhodococcus* sp. NCIMB 9784 [33] was fused to the C-terminus of OleT_{JE}. In the presence of the electron donor NADPH, the fusion protein OleT_{JE}-RhFRED successfully converted lauric acid (C₁₂) into 1-undecene with the conversion ratio of 51.1 \pm 0.3%, approximately half that of OleT_{JE} plus H₂O₂ (93.0 \pm 4.3% conversion; Figure 2). Notably, the product distribution (decarboxylation *versus* hydroxylation) of the OleT_{JE}-RhFRED catalyzed reaction was similar to that of the OleT_{JE} reaction (Figure 1B), indicating the fused reductase domain has no significant impact on the catalytic mechanism of P450 domain. As control, OleT_{JE} cannot directly use NADPH as electron donor. OleT_{JE}-RhFRED was not active by itself. Interestingly, OleT_{JE}-RhFRED retained its ability to use H₂O₂ as a cofactor, but there was not a significant additive effect between NADPH and H₂O₂ for supporting the activity of OleT_{JE}-RhFRED (Figure 2).

To prevent the spontaneous generation of H₂O₂ during the OleT_{JE}-RhFRED reaction, which could complicate interpretation of the results, dithiothreitol (DTT: the reducing agent often used in the storage and reaction buffer of P450 proteins) was omitted from all buffers used during protein purification, storage and reaction because it has previously been reported that DTT, dioxygen and the heme iron center of P450 can react to generate H₂O₂ [16,42]. To further exclude the possibility that H₂O₂ could be produced from the peroxide shunt pathway during the monooxygenase catalytic cycle [27], bovine liver catalase that is able to efficiently eliminate H₂O₂ was added to the fatty acid decarboxylation reactions (in the DTT-free buffer) catalyzed by OleT_{JE} and OleT_{JE}-RhFRED. In the OleT_{JE} plus H₂O₂ system, pre-addition of catalase completely abolished the reaction (Figure 3). By contrast, in the OleT_{JE}-RhFRED plus NADPH reaction, pre-added catalase did not significantly change (indeed, it slightly improved) the conversion of

myristic acid to 1-tridecene (Figure 3). This clearly indicates that the decarboxylation activity of OleT_{JE}-RhFRED can be solely supported by NADPH.

Substrate specificity of OleT_{JE} and OleT_{JE}-RhFRED

The substrate preference of OleT_{JE} and OleT_{JE}-RhFRED provides useful information to help understand the *in vivo* behavior of these fatty acid decarboxylases and to guide the metabolic engineering of fatty acid biosynthesis. Thus, we determined their substrate specificity (Figure 4) using a number of straight-chain saturated fatty acids with even-numbered chain length ranging from C₈ to C₂₀. In the case of OleT_{JE} (Figure 4A), myristic acid (C₁₄) turned out to be the best substrate for olefin production among the tested fatty acids with the conversion ratio of 97.0 \pm 2.8%. Lauric acid (C₁₂) and palmitic acid (C₁₆) were sub-optimal. OleT_{JE} was only able to convert a minority of stearic acid (C₁₈) and arachidic acid (C₂₀), but could not decarboxylate capric acid (C₁₀) or caprylic acid (C₈).

In comparison, the substrate preference spectrum of OleT_{JE}-RhFRED slightly shifted toward shorter fatty acids (Figure 4B), which suggests that the P450-reductase interaction might induce small conformational change of the OleT_{JE} active site. Specifically, OleT_{JE}-RhFRED showed the highest activity against lauric acid (83.8 \pm 0.1% conversion), but was inactive toward arachidic acid. Similar to OleT_{JE}, OleT_{JE}-RhFRED could not decarboxylate capric acid or caprylic acid. It is also worth noting that the system of OleT_{JE}-RhFRED plus NADPH plus O₂ was less active than OleT_{JE} plus H₂O₂ when reacting with most tested fatty acids except for lauric acid, suggesting OleT_{JE} might have evolved to become a better peroxygenase than a monooxygenase.

In vivo production of α -alkenes in *Escherichia coli*

Motivated by the efficient *in vitro* conversion of free fatty acids (C_n) to corresponding α -alkenes (C_{n-1}) by two related but mechanistically distinct decarboxylation systems

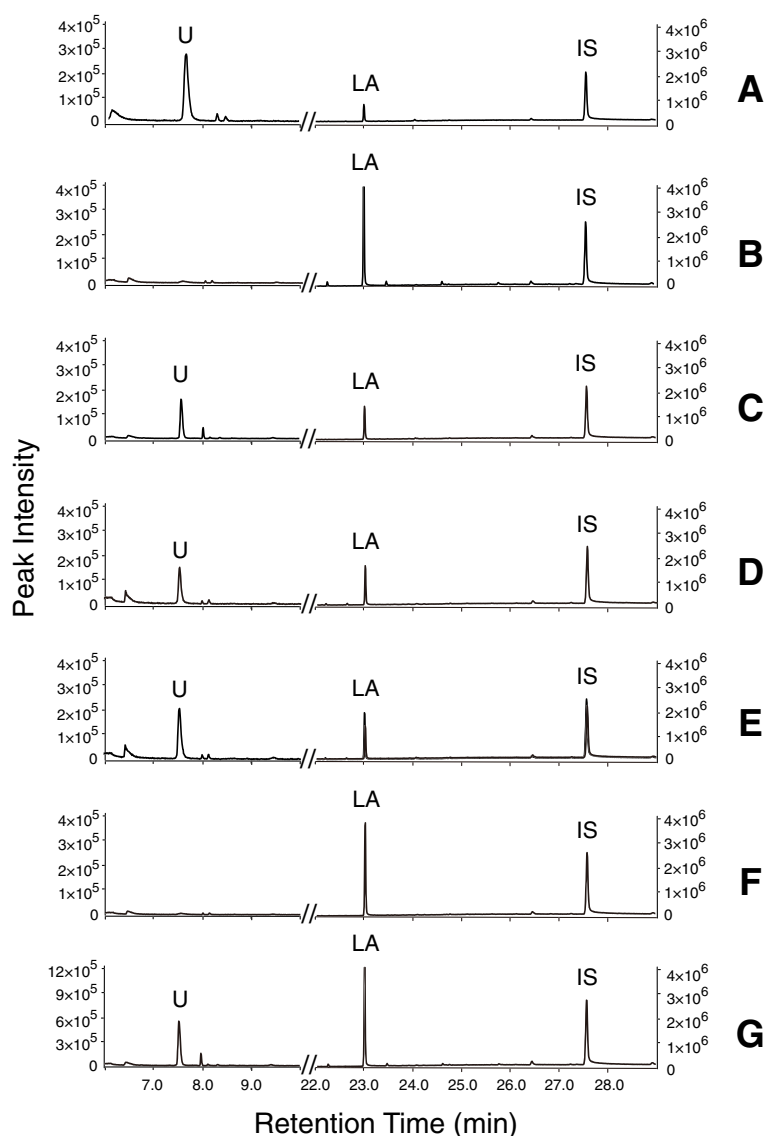


Figure 2 Gas chromatography-mass spectroscopy analysis of decarboxylation reactions (10 min) catalyzed by OleT_{JE} or OleT_{JE}-RhFRED under different reaction systems. (A) OleT_{JE} + H₂O₂; (B) OleT_{JE} + NADPH; (C) OleT_{JE}-RhFRED + NADPH; (D) OleT_{JE}-RhFRED + H₂O₂; (E) OleT_{JE}-RhFRED + NADPH + H₂O₂; (F) OleT_{JE}-RhFRED in absence of NADPH; (G) authentic standards of 1-undecene (U) lauric acid (LA) and heptadecanoic acid (IS: internal standard).

including OleT_{JE} plus H₂O₂ and OleT_{JE}-RhFRED plus NADPH plus O₂, we sought to engineer hydrocarbon-producing strains of *E. coli*. To guarantee sufficient substrate supply of free fatty acids for OleT_{JE} P450 decarboxylase, two previously engineered fatty acid-overproducing strains including XL100 (BL21:Δ*fadD*) and XL100/(pMSD8 + pMSD15) [43] were used as hosts for the decarboxylases. Transformation of these two strains with pET28b-*oleT_{JE}* or pET28b-*oleT_{JE}*-RhFRED resulted in YL5 (XL100 with pET28b-*oleT_{JE}*), YL6 (XL100 with pET28b-*oleT_{JE}*-RhFRED), YL7 (XL100 with pET28b-*oleT_{JE}*, pMSD8 and pMSD15), and YL8 (XL100 with pET28b-*oleT_{JE}*-RhFRED, pMSD8 and pMSD15) (Table 1).

As expected, all these engineered strains successfully accumulated multiple α -alkenes, including 1-tridecene, trideca-1,6-diene, 1-pentadecene, pentadecene-1,8-diene and heptadeca-1,10-diene (Figure 5A and Additional file 4: Figure S4), which correspond to the decarboxylation products of the 14:0, 14:1, 16:0, 16:1 and 18:1 fatty acids, respectively. This fatty acid composition is consistent with previous reports [43]. In all cases, the major alkene product was heptadeca-1,10-diene with the highest yield of 7.7 mg·l⁻¹ in YL7. Since XL100/(pMSD8 + pMSD15) is able to produce more free fatty acids than XL100 [43], the total alkene titers of YL7 and YL8 were significantly greater than those of YL5 and YL6. Notably, expression

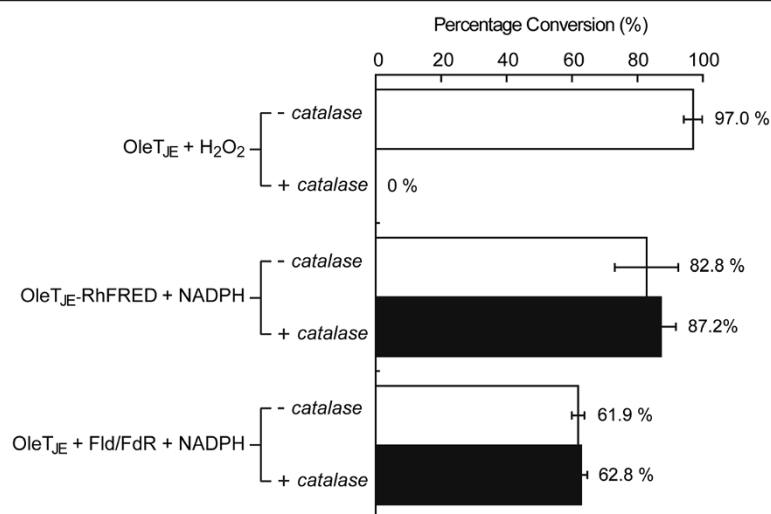


Figure 3 Effects of catalase on the *in vitro* decarboxylation activity of three OleT_{JE} (1 μM) reaction systems. Systems comprised OleT_{JE} plus H₂O₂, OleT_{JE}-RhFRED plus NADPH, and OleT_{JE} plus flavodoxin and flavodoxin reductase plus NADPH. The percentage conversion of myristic acid is shown beside each bar.

of P450 decarboxylases did not significantly affect the cell growth of all strains (Additional file 5: Figure S5).

According to a previous report [44], H₂O₂ levels in growing *E. coli* are less than 20 nM, and 2 μM H₂O₂ could cause substantial growth inhibition. Therefore, the physiological concentration of H₂O₂ is far below the optimal H₂O₂ concentration (200 to 500 μM) [16] for supporting OleT_{JE} catalysis. Thus, we had predicted that OleT_{JE}-RhFRED using endogenous NADPH, whose physiological concentration is around 120 μM [45], might catalyze the decarboxylation more efficiently than OleT_{JE} using H₂O₂ as a cofactor. Unexpectedly, the highest titer (11.8 mg·l⁻¹) and productivity (4.5 mg·g⁻¹·dcw⁻¹) of total alkenes was achieved by YL7 with OleT_{JE} instead of YL8 with OleT_{JE}-RhFRED. This contradiction has led to a hypothesis that OleT_{JE} is able to employ the

native redox system of *E. coli* to drive catalysis, such as P450_{CLA} and P450_{BSβ} [41].

To test this hypothesis, we expressed and purified the *E. coli* Fld and FdR. In the *in vitro* reaction containing OleT_{JE}, Fld, FdR and NADPH, myristic acid was substantially converted into 1-tridecene (Figure 3). Therefore, Fld and FdR probably better serve OleT_{JE} than the fused RhFRED *in vivo*, explaining why YL7 was a better alkene producer than YL8. However, the supportive role of H₂O₂ cannot be entirely neglected.

Inspired by these results, we attempted to enhance the level of dissolving dioxygen for improving OleT_{JE} catalysis during the cultivation of the best alkene producer, YL7, by applying a higher rotation rate of 250 rpm. Moreover, the defined mineral medium [14] containing 3% glucose was used to standardize the culture condition for

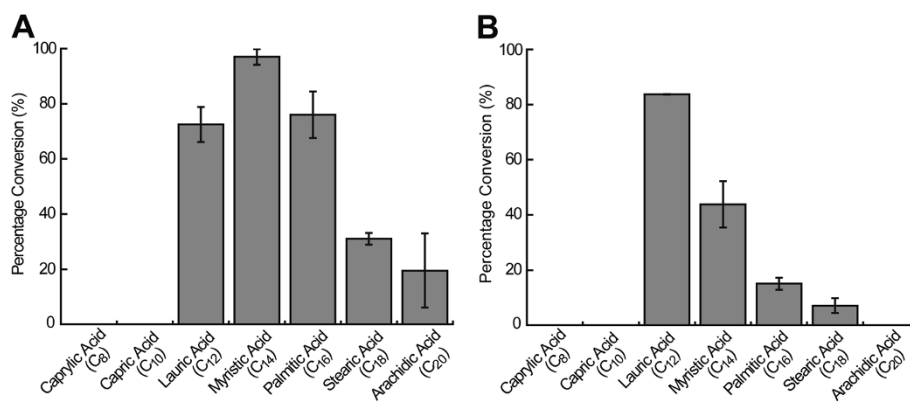


Figure 4 Substrate preference spectrum of (A) OleT_{JE} and (B) OleT_{JE}-RhFRED. The substrate preference was determined by calculating the percentage conversion of each fatty acid substrate into corresponding α-alkene product. In these assays, 0.2 μM enzymes were used.

Table 1 Strains and plasmids used in this study

Strain or plasmid	Relevant characteristics	Source or reference
<i>E. coli</i> strains		
BL21(DE3)	F ⁻ ompT gal dcm lon hsdSB (r _B m _B) λ(DE3)	Novagen
DH5α	F ⁻ λ80lacZΔM15 Δ (ΔlacZYA-argF) U169 recA1 endA1 hsdR17 (rk ⁻ , mk ⁺) phoA supE44 thi-1 gyrA96 relA1 λ ⁻	Invitrogen
XL100	BL21: Δ <i>fadD</i> ^a	[43]
YL1	BL21(DE3) with pET28b- <i>oleT_{JE}</i>	This study
YL2	BL21(DE3) with pET28b- <i>oleT_{JE}</i> -RhFRED	This study
YL3	BL21(DE3) with pACYCDuet- <i>fdR</i>	This study
YL4	BL21(DE3) with pCDFDuet- <i>fld</i>	This study
YL5	XL100 with pET28b- <i>oleT_{JE}</i>	This study
YL6	XL100 with pET28b- <i>oleT_{JE}</i> -RhFRED	This study
YL7	XL100 with pET28b- <i>oleT_{JE}</i> , pMSD8 ^b , pMSD15 ^c	This study
YL8	XL100 with pET28b- <i>oleT_{JE}</i> -RhFRED, pMSD8, pMSD15	This study
<i>Jeotgalicoccus</i> sp. ATCC 8456	Wild type	ATCC
Plasmids		
pET28b	Km ^r , T7 promoter, pBR322 origin	Novagen
pET28b- <i>oleT_{JE}</i>	Km ^r , pET28b derivative containing <i>oleT_{JE}</i> gene	This study
pET28b- <i>oleT_{JE}</i> -RhFRED	Km ^r , pET28b derivative containing <i>oleT_{JE}</i> and RhFRED gene	This study
pACYCDuet-1	Cm ^r , T7 promoter, P15A origin	Novagen
pACYCDuet- <i>fdR</i>	Cm ^r , pACYCDuet-1 derivative containing <i>fdR</i> gene	This study
pCDFDuet-1	Str ^r , T7 promoter, CloDF13 origin	Novagen
pCDFDuet- <i>fld</i>	Str ^r , pCDFDuet-1 derivative containing <i>fld</i> gene	This study
pMSD8	Amp ^r , P _{T7} : <i>accB</i> , C, D, A, pSC101 origin	[43]
pMSD15	Cm ^r , P _{BAD} : <i>tesA</i> , p15a origin	[43]

^aThe *fadD* gene encodes the acyl-CoA synthetase, which is the first enzyme involved in fatty acid degradation. ^bpMSD8: the expression vector for *E. coli* acyl-CoA carboxylase. ^cpMSD15: the plasmid used for overexpression of the *E. coli* thioesterase gene *tesA*. Amp, ampicillin; Cm, chloramphenicol; Km, kanamycin; Str, streptomycin.

the future metabolic engineering, and the culture time was extended to 40 h. Significantly, the total alkene titer of YL7 under these conditions was 97.6 mg·l⁻¹ (Figure 5B), which is almost seven-fold higher than that in lysogeny broth (LB) medium at 220 rpm for 20 h (Figure 5A). The productivity of total alkenes was also improved from 4.5 mg·g dcw⁻¹ (in LB over 20 h) to 24.9 mg·g dcw⁻¹ (in the defined mineral medium over 40 h, data not shown). Again, heptadeca-1,10-diene was the major alkene product (41.4 mg·l⁻¹). As control, the production of free fatty acids by the strain XL100/(pMSD8 + pMSD15) without OleT_{JE}

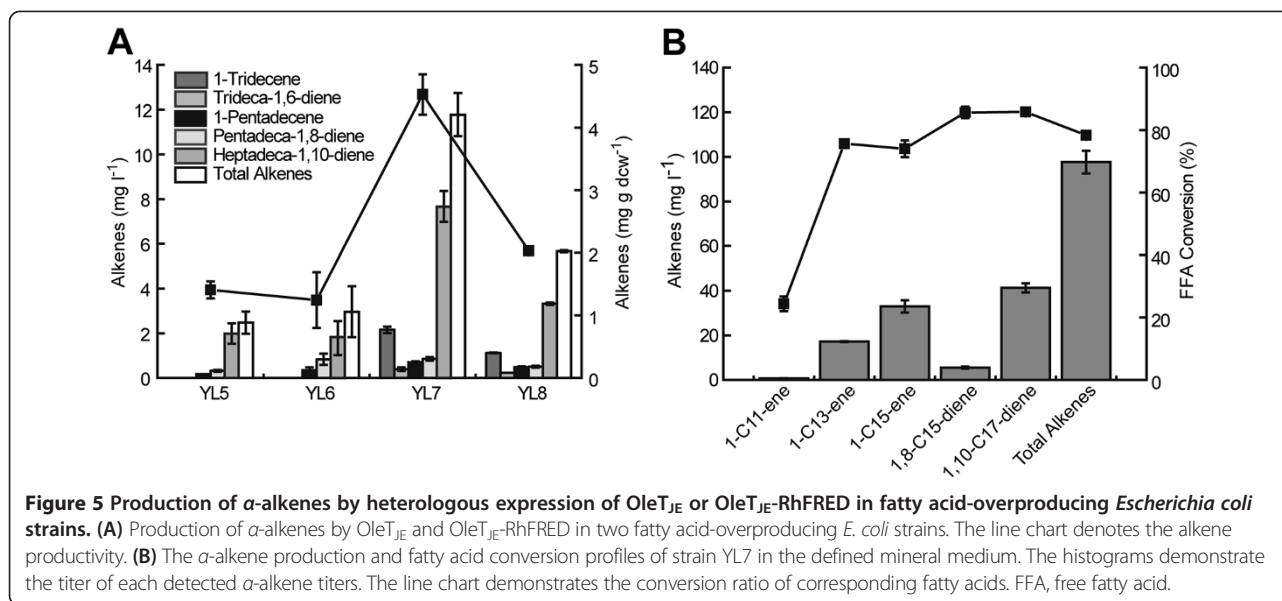
expression was evaluated. Octadec-11-enoic acid was the most abundant fatty acid with a titer of 96.1 mg·l⁻¹ (Additional file 6: Figure S6). This well explains why its decarboxylated product, heptadeca-1,10-diene, had the highest yield among produced alkenes (Figure 5). With respect to fatty acid conversion, octadec-11-enoic acid had the highest conversion ratio (85.9%) followed by hexadec-9-enoic acid (85.7%), myristic acid (75.7%), palmitic acid (74.0%) and lauric acid (24.4%). The low conversion of lauric acid seems to be inconsistent with the *in vitro* result (Figure 4A), which is likely due to the low intracellular concentration of the C₁₂ fatty acid.

Discussion

The cytochrome P450 enzymes are a superfamily of b-type heme proteins, capable of catalyzing more than 20 different types of reactions [25,46,47]. The H₂O₂-assisted decarboxylation of long-chain fatty acids catalyzed by P450 OleT_{JE} represents a novel activity of this highly versatile superfamily. This activity may be mechanistically similar to that of P450_{Rm} (CYP53B) from the yeast *Rhodotorula minuta* [48], which decarboxylates isovalerate to form isobutene.

According to protein sequence alignment (Additional file 3: Figure S3), OleT_{JE} belongs to the CYP152 peroxygenase family together with the well-studied P450_{BSβ} and P450_{SPα} [28,29], and other members. Functionally, these P450 peroxygenases were previously thought to have no ability of utilizing dioxygen to drive catalysis as typical P450 monooxygenases do [39]. However, we have unambiguously demonstrated that OleT_{JE} can perform H₂O₂-independent catalysis when partnering with the fused RhFRED reductase or the *E. coli* Fld/FdR system to transfer electrons from NADPH to the heme iron reactive center. This strongly suggests that OleT_{JE} can undergo the monooxygenase catalytic cycle to generate the highly reactive ferryl-oxo cation radical species (Compound I) for catalysis. In the well-accepted mechanism for dioxygen activation in P450 monooxygenases (Figure 6), two protons (and two electrons from NADPH) are required for generation of Compound I [27]. However, the conserved threonine and an acidic residue involved in proton delivery in normal P450 monooxygenases [34,40] are replaced by Pro and Arg, respectively, which are absolutely conserved in OleT_{JE} and all other known CYP152 family members (Additional file 3: Figure S3). This strongly suggests an unknown proton transfer pathway. To elucidate this hypothetical pathway, we are currently seeking to solve the crystal structure of OleT_{JE}.

Evolutionarily, because the early Earth's environment probably had more H₂O₂ and peroxygenated organic chemicals than O₂, P450 peroxygenases are presumed to have emerged ahead of P450 monooxygenases [38]. Thus, the ability of most P450 monooxygenases to use



H_2O_2 as a surrogate for the $O_2/2e^-/2H^+$ system (peroxide shunt pathway, Figure 6) could be understood as a remnant function inherited from their peroxygenase ancestors, whereas the $OleT_{JE}$ P450 peroxygenase bearing the monooxygenase property might represent a transition species during the evolving process from peroxygenase to monooxygenase.

Under certain circumstances, such as *in vitro* synthetic reactions using purified P450s and application of P450 enzymes in laundry detergents, the peroxygenase activity supported by H_2O_2 is advantageous because expensive redox partner proteins and NAD(P)H are not needed

[30,31]. However, the peroxygenase nature is not a good feature if attempting to construct a biofuel-producing microorganism by taking advantage of the fatty acid decarboxylation activity of $OleT_{JE}$. Essentially, the intracellular level of H_2O_2 cannot be increased to the concentration (10^1 to 10^2 μM range) required for efficiently supporting $OleT_{JE}$ because at this level, H_2O_2 is toxic or fatal to all organisms including *E. coli*. Thus, that the activity of P450 $OleT_{JE}$ can be supported by O_2 /redox partner(s)/NADPH besides H_2O_2 is a significant discovery because the former three factors are more viable targets for metabolic engineering [49-51]. Being aware of this,

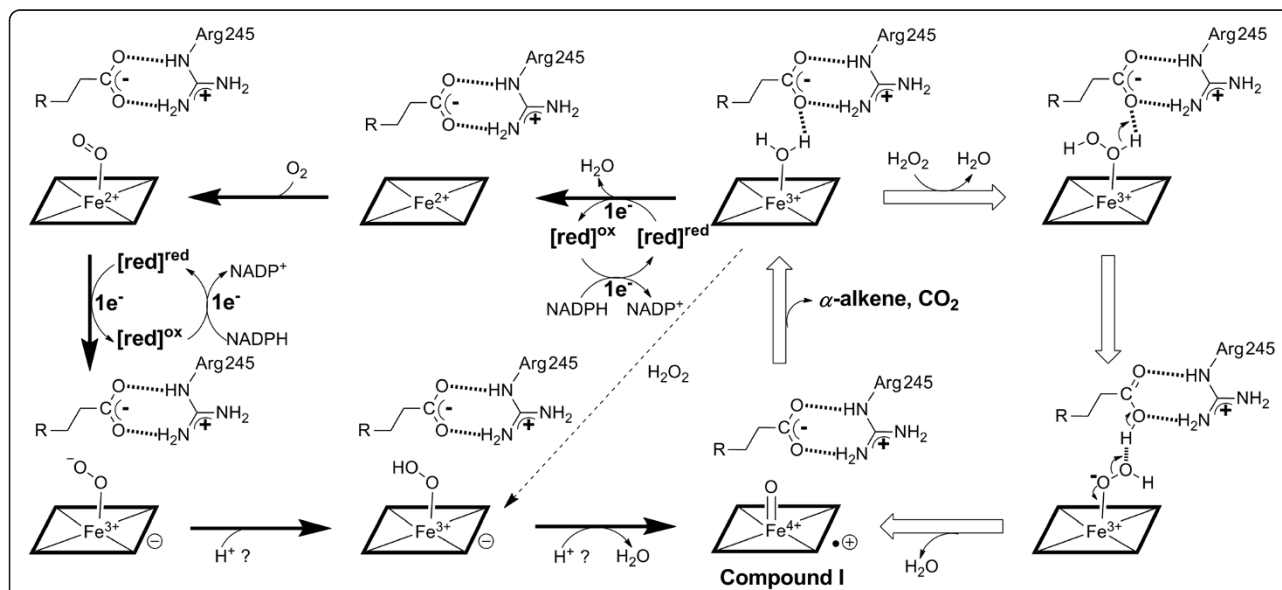


Figure 6 Proposed two alternative catalytic mechanisms of $OleT_{JE}$ with (monooxygenase-like) or without (peroxygenase-like) redox systems. The Arg²⁴⁵ is proposed to be required for substrate anchoring via the strong electrostatic interactions with the carboxyl group of fatty acid substrate. The supply of two protons in the putative monooxygenase catalytic cycle remains unclear. The dashed arrow indicates the peroxide shunt pathway.

future metabolic engineering work aiming to improve the *in vivo* productivity of α -alkenes by OleT_{JE} probably should be directed to improvement of the intracellular level of dioxygen, redox partner protein(s) and/or NADPH. For example, among numerous metabolic strategies for *in vivo* up-regulation of NADPH [49], the NADPH regenerating system could be used for maintaining the high intracellular level of this reducing cofactor to better support the activity of OleT_{JE} and the overproduction of fatty acids [52,53], both of which require a sufficient supply of NADPH.

In the mutant *E. coli* strains with up-regulated fatty acid biosynthesis (XL100 and XL100/(pMSD8 + pMSD15)), both OleT_{JE} and OleT_{JE}-RhFRED were functional, therefore significantly converting fatty acids into α -alkenes. Consistent with the previous report [16], the common major product of four engineered alkene producers (YL5-8) turned out to be heptadeca-1,10-diene, but the second most abundant alkene varied (Figure 5A). This is likely because octadec-11-enoic acid (the precursor of heptadeca-1,10-diene) was the predominant component of the fatty acid pool of the tested strains (Additional file 6: Figure S6). However, there might be an additional reason that octadec-11-enoic acid is a preferred substrate *in vivo*. Interestingly, in previous studies [43,54,55], palmitic acid rather than octadec-11-enoic acid was the major component in the fatty acid pool of *E. coli*. This inconsistency could be due to different culture conditions.

After 20 h cultures of YL7 in LB medium, significant amounts of fatty acids remained unreacted with OleT_{JE} (Additional file 4: Figure S4). By contrast, the majority of fatty acids in YL7 were consumed by OleT_{JE} over the longer and more oxygenated cultivation in the defined mineral medium (Figure 5B). This suggests that, at the current stage, the yield of fatty acids might be the major limiting factor for further improvement of α -alkene titers. Thus, the overproduction of fatty acids needs to be significantly optimized prior to other engineering efforts on up-regulating the level of OleT_{JE}, redox partners, O₂ and NADPH.

In this report, the best bio-hydrocarbon-producing strain YL7 accumulated 97.6 mg·l⁻¹ of total alkenes. This yield is comparable to or better than a majority of engineered alkane and alkene biosynthetic pathways with reported yields. These include the artificial alkane biosynthetic pathway in *E. coli* consisting of the carboxylic acid reductase from *Mycobacterium marinum* that catalyzes formation of fatty aldehydes directly from fatty acids, and the aldehyde decarbonylase from *Synechocystis* sp. PCC 6803 to produce alkanes (yield: approximately 2 mg·l⁻¹) [56]; the hybrid system using the fatty acid reductase complex (LuxC, LuxE and LuxD) to provide fatty aldehyde as the substrate for downstream aldehyde decarbonylase to generate alkanes (yield: ap-

proximately 2 to 5 mg·l⁻¹) [20]; and the ATP required decarboxylation of 3-hydroxy-3-methylbutyrate catalyzed by the R74H mutant of mevalonate diphosphate decarboxylase from *Saccharomyces cerevisiae* (productivity: 5,888 pmol·h⁻¹·g cells⁻¹) [57]. It is only lower than the 300 mg·l⁻¹ of total alkane titer produced when the cyanobacterial pathway consisting of the acyl-ACP reductase Orf1594 from *Synechococcus elongates* PCC7942 and the aldehyde decarbonylase from *Nostoc punctiforme* PCC73102 was heterologously expressed in *E. coli* [14]. However, it is expected that more metabolic engineering efforts and optimization of fermentation will further increase the total alkene titers of the OleT_{JE}/*E. coli* system, which is currently ongoing in our laboratory.

Conclusions

The H₂O₂ independence of OleT_{JE} described in this work not only raises a number of fundamental questions regarding its monooxygenase-like mechanism, but also could direct future metabolic engineering work toward improvement of O₂/redox partner(s)/NADPH for optimal activity of OleT_{JE} *in vivo*. Considering its high conversion rate *in vitro* and H₂O₂-independent functionality *in vivo*, it is of great potential for engineering a hyper-producer of α -alkenes on the basis of OleT_{JE}.

Methods

Materials

Fatty acid substrates, terminal alkene authentic standards, and derivatizing reagents were purchased from TCI (Shanghai, China). Antibiotics were obtained from Solar-Bio (Beijing, China). Other chemicals were from Sigma Aldrich (St. Louis, MO, USA) or Ameresco (Solon, OH, USA). Oligonucleotides were synthesized by Sangon Biotech (Shanghai, China), and their sequences are shown in Additional file 7: Table S1. The *Pfu* DNA polymerases and all restriction endonucleases were obtained from Fermentas (Vilnius, Lithuania) or Takara (Dalian, China). The kits used for molecular cloning were from OMEGA Bio-Tek (Jinan, China) or Promega (Madison, WI, USA). Protein purification used Qiagen Ni-NTA resin (Valencia, CA, USA), Millipore Amicon Ultra centrifugal filters (Billerica, MA, USA) and PD-10 desalting columns from GE Healthcare (Piscataway, NJ, USA). Bovine liver catalase was purchased from Sigma Aldrich.

Molecular cloning

Strains and plasmids constructed and used in this study are listed in Table 1. The gene of *oleT_{JE}* was amplified from the genomic DNA of *Jeotgalicoccus* sp. ATCC 8456 using the primer pair of OleT-NdeI/OleT-HindIII (Table 1). The gel-cleaned PCR fragment was double digested by *NdeI* and *HindIII* and subsequently ligated into the *NdeI/HindIII* pre-treated pET28b to afford

pET28b-*oleT_{JE}* (Additional file 8: Figure S7A). For pET28b-*oleT_{JE}-RhFRED*, the genes of *oleT_{JE}* and *RhFRED* were first fused by overlap extension PCR [58]. Briefly, the gene encoding RhFRED reductase was amplified from the previously constructed pET28b-*pikC-RhFRED* [59,60] with a pair of primers including RhFRED-F and RhFRED-R. The *oleT_{JE}* gene was amplified from pET28b-*oleT_{JE}*, using the primers OleT-F and OleT-RhFRED-OE. Then the two PCR fragments with overlap sequence were mixed, annealed, extended and finally amplified with the OleT-F/RhFRED-R primer pair, giving rise to the fused gene of *oleT_{JE}-RhFRED*. This fusion product was digested by *NdeI/HindIII* and inserted into the *NdeI/HindIII*-digested pET28b to generate pET28b-*oleT_{JE}-RhFRED* (Additional file 8: Figure S7B).

Using the genomic DNA of *E. coli* DH5 α as template, the two genes encoding Fld and FdR were amplified with the primer pairs of Fld-BamHI-F/Fld-SalI-R and FdR-BamHI-F/FdR-SalI-R, respectively. Next, the *fld* and *fdR* genes were sub-cloned into pACYCDuet-1 and pCDFDuet-1 respectively, resulting in pACYCDuet-*fld* and pCDFDuet-*fdR*. All sub-cloned sequences were confirmed by DNA sequencing at Sangon Biotech, Shanghai.

Protein overexpression and purification

The *E. coli* BL21(DE3) cells carrying the recombinant expression vector were grown at 37°C for 16 to 20 h in LB medium containing certain selective antibiotics (50 $\mu\text{g}\cdot\text{ml}^{-1}$ kanamycin, 34 $\mu\text{g}\cdot\text{ml}^{-1}$ chloramphenicol, or 50 $\mu\text{g}\cdot\text{ml}^{-1}$ streptomycin), which were used to inoculate (1:100 ratio) Terrific Broth medium containing corresponding antibiotics, thiamine (1 mM), 10% glycerol and a rare salt solution [61]. Cells were grown at 37°C for 3 to 4 h until the optical density at 600 nm (OD₆₀₀) reached 0.6 to 0.8, at which isopropyl- β -D-thiogalactopyranoside (IPTG, 0.2 mM as final concentration) and δ -aminolevulinic acid (0.5 mM, only for P450 expression) were added, followed by 18 h of cultivation at 18°C.

Protein purification was carried out as described elsewhere [61] with slight modifications. Specifically, the cell pellets harvested by centrifugation (5,000 \times g, 4°C, 15 min) were stored at -80°C and melted at ambient temperature immediately before use. Then the cell pellets were resuspended in 40 ml of pre-chilled lysis buffer (pH 8.0, 50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol and 10 mM imidazole) through vortexing. Following a sonication step, the cell lysate was centrifuged at 12,000 \times g for 30 min to remove the insoluble fraction. To the supernatant, 1 ml of Ni-NTA resin was added and gently mixed at 4°C for 1 h. The slurry was loaded onto an empty column, and washed with approximately 100 ml of wash buffer (pH 8.0, 50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol and 20 mM

imidazole) until no proteins were detectable in flow-through. The bound target proteins were eluted with elution buffer (pH 8.0, 50 mM NaH₂PO₄, 300 mM NaCl, 10% glycerol and 250 mM imidazole). The eluent was concentrated with an Amicon Ultra centrifugal filter, and buffer exchanged on a PD-10 desalting column. Finally, the desalted purified proteins (Additional file 1: Figure S1) in storage buffer (pH 7.4, 50 mM NaH₂PO₄, 10% glycerol) were flash-frozen by liquid nitrogen and stored at -80°C for later use.

Determination of protein concentration

Following the method described by Omura and Sato [62], the CO-bound reduced difference spectrum (Additional file 2: Figure S2) of each P450 enzyme was recorded on a UV-visible spectrophotometer DU 800 (Beckman Coulter, Fullerton, CA, USA). The functional P450 concentration was calculated using the extinction coefficient ($\epsilon_{450-490}$) of 91,000 M⁻¹·cm⁻¹. The concentration of Fld and FdR from *E. coli* was determined using $\epsilon_{579} = 4,570$ M⁻¹·cm⁻¹ [63] and $\epsilon_{456} = 7,100$ M⁻¹·cm⁻¹ [64], respectively.

In vitro enzymatic assays with purified proteins

The fatty acid decarboxylation assays containing 0.2 to 1.0 μM OleT_{JE} or OleT_{JE}-RhFRED, 200 μM fatty acid substrate (from C₈ to C₂₀), 500 μM H₂O₂ (for OleT_{JE}) or 500 μM NADPH (for OleT_{JE}-RhFRED) in 200 μl of storage buffer were carried out at 28°C for 2 h. In the assay to test whether *E. coli* Fld and FdR are able to support the activity of OleT_{JE}, 5 μM Fld and 5 μM FdR were mixed with 200 μM myristic acid, 500 μM NADPH and 1 μM OleT_{JE} in 200 μl of storage buffer. To remove spontaneously generated H₂O₂, bovine liver catalase was added to the final concentration of 20 U·ml⁻¹.

All above described reactions were quenched by addition of 20 μl of 10 M HCl. Heptadecanoic acid was added as internal standard and the mixture was extracted by 200 μl ethyl acetate. Samples were then analyzed by gas chromatography-mass spectroscopy (GC-MS; see below).

In vivo production of α -alkenes

The *E. coli fadD* deletion mutant strain XL100 [43] that overproduces fatty acids was selected as the starting host for construction of alkene-producing strains. Plasmids pMSD8 and pMSD15 were gifts from Dr. John Cronan and were used in the strain XL100 to construct fatty acid-overproducing strains. Bacterial cells transformed with certain plasmid(s) (Table 1) were either grown in 50 ml of LB medium or in 50 ml of defined mineral medium [14] containing 3% glucose as the carbon source, supplemented with appropriate selective antibiotics, thiamine (1 mM) and a rare salt solution. All cultivations were performed at 37°C and induced at an OD₆₀₀ of 0.9 to 1.0 with 0.4% arabinose (for pMSD15)

followed by 0.2 mM IPTG (for pMSD8, and pET28b-*oleT_{JE}* or pET28b-*oleT_{JE}-RhFRED*) after 0.5 h. Next, cells were grown at 28°C (the optimal temperature for OleT_{JE} expression) for an additional 20 h at 220 rpm (in LB medium) or 40 h at 250 rpm (in the defined mineral medium). For analysis of hydrocarbon production, 20 ml of culture with 10 µl heptadecanoic acid added as internal standard was sonicated for 10 min and then thoroughly mixed with an equal volume of chloroform-methanol (2:1, vol/vol). The aqueous-organic mixture was centrifuged (8,000 × g for 15 min) for phase separation. The organic phase was transferred into a clean tube, evaporated under a nitrogen flow, and re-dissolved in 500 µl of n-hexane as the testing sample. Prior to GC-MS analysis, 5 µl eicosane was added as calibration standard. All experiments were repeated two to four times.

Analytical methods

The GC-MS analytical method for hydrocarbon and fatty acid samples was adapted from Guan *et al.* [65]. The analyses were performed on an Agilent 7890A gas chromatograph equipped with a capillary column HP-INNOWAX (Agilent Technologies, Santa Clara, CA, USA; cross-linked polyethylene glycol, i.d. 0.25 µm film thickness, 30 m by 0.25 mm) coupled to an Agilent 5975C MSD single quadrupole mass spectrometer operated under electron ionization mode at 70 eV in the scan range of 50 to 500 m/z. The helium flow rate was set to 1 ml·min⁻¹. The oven temperature was controlled initially at 40°C for 4 min, then increased at the rate of 10°C per min to 250°C, and held for 15 min. The injecting temperature was set to 280°C with the injection volume of 1 µl under splitless injection conditions. Under these conditions, the previously reported thermal degradations of α- and β-hydroxy fatty acids to form alkenes (the minor products of OleT_{JE} catalyzed reaction) in the GC inlet [16] were not observed (Additional file 9: Figure S8), making the silylating protection of the hydroxyl group unnecessary if only caring about the α-alkene products. To detect α- and β-hydroxy fatty acids, samples were derivatized with an equal volume of *N*, *O*-bis(trimethylsilyl)trifluoroacetamide with 1% trimethylchlorosilane at 70°C for 15 min. GC-MS analysis followed the previous protocol developed by Rude *et al.* [16] except for using the Agilent J&W DB-5 MS column (i.d. 0.25 µm film thickness, 50 m by 0.25 mm). During GC-MS analysis, peak identity was determined by comparison of retention time and fragmentation pattern with authentic standard compounds where available and to the National Institute of Standards and Technology, USA mass spectral database. The location of double bond(s) in α-olefins was deduced by derivatization with dimethyl disulfide as described previously [66].

Quantification was achieved by comparison of integrated peak areas with calibration curves of authentic standards. The conversion percentages of free fatty acids to corresponding α-alkenes were estimated using the equation: [total alkenes]/([total alkenes] + [total free fatty acids]).

Additional files

Additional file 1: Figure S1. SDS-PAGE analysis of purified (A) OleT_{JE} and (B) OleT_{JE}-RhFRED. M, protein marker.

Additional file 2: Figure S2. CO-bound reduced spectra of purified (A) OleT_{JE} and (B) OleT_{JE}-RhFRED.

Additional file 3: Figure S3. Protein sequence alignment of CYP152 family members and three selected P450 monooxygenases. Protein sequences were obtained from NCBI protein databases. CYP152A1 (P450_{B58} from *Bacillus subtilis*); CYP152A2 (P450_{CLA} from *Clostridium acetobutylicum*); CYP152B1 (P450_{SPa} from *Sphingomonas paucimobilis*); CYP152B2 (from *Azotobacter vinelandii*); CYP152C1 (from *Rhodobacter sphaeroides*); CYP152C2 (from *Rhodobacter sphaeroides*); CYP152D1 (from *Streptomyces scabies*); CYP152E1 (from *Cyanospora* sp. CCY0110); CYP107L1 (P450_{PKC} from *Streptomyces venezuelae*); CYP102A1 (P450_{BM3} from *Bacillus megaterium*); CYP101A1 (P450_{CAM} from *Pseudomonas putida*). Conserved amino acid residues are shaded. The Arg and Pro absolutely conserved in CYP152 family are marked by asterisks.

Additional file 4: Figure S4. GC-MS analysis of the organic extract of the YL7 culture in LB broth. Eicosane and heptadecanoic acid are served as calibration standard (CS) and internal standard (IS), respectively.

Additional file 5: Figure S5. The dry cell weight of the YL5-8 cultures using LB broth.

Additional file 6: Figure S6. Production profile of free fatty acids by the strain XL100/(pMSD8 + pMSD15).

Additional file 7: Table S1. Primers used in this study.

Additional file 8: Figure S7. Plasmid maps for (A) pET28b-*oleT_{JE}* and (B) pET28b-*oleT_{JE}-RhFRED* expression vectors.

Additional file 9: Figure S8. GC-MS analysis of the α- and β-hydroxy myristic acid authentic standards on HP-INNOWAX capillary column. (A) Without derivatization, α-hydroxy myristic acid was unseen due to its high boiling point. Importantly, no thermally degraded terminal olefin (1-tridecene) was observed in the dashed box. (B) Without derivatization, β-hydroxy myristic acid was unseen due to its high boiling point. Again, no thermally degraded terminal olefin (1-tridecene) was observed in the dashed box. (C) The decarboxylation reaction of myristic acid (2 h) catalyzed by OleT_{JE}. The peak shown in the dashed box corresponds to 1-tridecene.

Abbreviations

ACP: acyl carrier protein; CoA: coenzyme A; CYP: cytochrome P450 enzyme; DTT: dithiothreitol; FdR: flavodoxin reductase; Fld: flavodoxin; GC-MS: gas chromatography-mass spectrometry; IPTG: Isopropyl-β-D-thiogalactopyranoside; LB: lysogeny broth; OD: optical density; PCR: polymerase chain reaction; RhFRED: *Rhodococcus* fusion reductase.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

SL conceived of the study. YL, CW, XL and SL designed the experiments. YL, JY and WZ performed the experiments including plasmid construction, protein overexpression, purification, characterization and enzymatic assays. YL, CW and WG carried out GC-MS analysis. YL, CW and SL drafted the manuscript. JY, WZ, WG and XL helped to revise the manuscript. All authors read and approved the final manuscript.

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References

- Shafiee S, Topal E: **When will fossil fuel reserves be diminished?** *Energy Policy* 2009, **37**:181–189.
- Stephanopoulos G: **Challenges in engineering microbes for biofuels production.** *Science* 2007, **315**:801–804.
- Kerr RA: **Global warming is changing the world.** *Science* 2007, **316**:188–190.
- Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD: **Microbial engineering for the production of advanced biofuels.** *Nature* 2012, **488**:320–328.
- Lee SK, Chou H, Ham TS, Lee TS, Keasling JD: **Metabolic engineering of microorganisms for biofuels production: from bugs to synthetic biology to fuels.** *Curr Opin Biotechnol* 2008, **19**:556–563.
- Savage DF, Way J, Silver PA: **Defossilizing fuel: how synthetic biology can transform biofuel production.** *ACS Chem Biol* 2008, **3**:13–16.
- Demain AL: **Biosolutions to the energy problem.** *J Ind Microbiol Biotechnol* 2009, **36**:319–332.
- Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD: **Biofuel alternatives to ethanol: pumping the microbial well.** *Trends Biotechnol* 2008, **26**:375–381.
- Li H, Cann AF, Liao JC: **Biofuels: biomolecular engineering fundamentals and advances.** *Annu Rev Chem Biomol Eng* 2010, **1**:19–36.
- Wackett LP: **Biomass to fuels via microbial transformations.** *Curr Opin Chem Biol* 2008, **12**:187–193.
- Zhang F, Rodriguez S, Keasling JD: **Metabolic engineering of microbial pathways for advanced biofuel production.** *Curr Opin Biotechnol* 2011, **22**:775–783.
- Qiu Y, Tittiger C, Wicker-Thomas C, Le Goff G, Young S, Wajnberg E, Fricaux T, Taquet N, Blomquist GJ, Feyereisen R: **An insect-specific P450 oxidative decarboxylase for cuticular hydrocarbon biosynthesis.** *Proc Natl Acad Sci USA* 2012, **109**:14858–14863.
- Niehaus TD, Okada S, Devarenne TP, Watt DS, Sviripa V, Chappell J: **Identification of unique mechanisms for triterpene biosynthesis in *Botryococcus braunii*.** *Proc Natl Acad Sci USA* 2011, **108**:12260–12265.
- Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB: **Microbial biosynthesis of alkanes.** *Science* 2010, **329**:559–562.
- Beller HR, Goh EB, Keasling JD: **Genes involved in long-chain alkene biosynthesis in *Micrococcus luteus*.** *Appl Environ Microbiol* 2010, **76**:1212–1223.
- Rude MA, Baron TS, Brubaker S, Alibhai M, del Cardayre SB, Schirmer A: **Terminal olefin (1-alkene) biosynthesis by a novel P450 fatty acid decarboxylase from *Jeotgalicoccus* species.** *Appl Environ Microbiol* 2011, **77**:1718–1727.
- Alibhai MF, Rude MA, Schirmer A: **Methods and compositions for producing olefins.** 20110196180 A1 (Patent) 2012.
- Mendez-Perez D, Begemann MB, Pfleger BF: **Modular synthase-encoding gene involved in α -olefin biosynthesis in *Synechococcus* sp. strain PCC 7002.** *Appl Environ Microbiol* 2011, **77**:4264–4267.
- Gu L, Wang B, Kulkarni A, Gehret JJ, Lloyd KR, Gerwick L, Gerwick WH, Wipf P, Håkansson K, Smith JL, Sherman DH: **Polyketide decarboxylative chain termination preceded by *O*-sulfonation in curacin A biosynthesis.** *J Am Chem Soc* 2009, **131**:16033–16035.
- Howard TP, Middelhaufe S, Moore K, Edner C, Kolak DM, Taylor GN, Parker DA, Lee R, Smirnov N, Aves SJ, Love J: **Synthesis of customized petroleum-replica fuel molecules by targeted modification of free fatty acid pools in *Escherichia coli*.** *Proc Natl Acad Sci USA* 2013, **110**:7636–7641.
- Lennen RM, Pfleger BF: **Engineering *Escherichia coli* to synthesize free fatty acids.** *Trends Biotechnol* 2012, **30**:659–667.
- Youngquist JT, Lennen RM, Ranatunga DR, Bothfeldt WH, Mamer WD II, Pfleger BF: **Kinetic modeling of free fatty acid production in *Escherichia coli* based on continuous cultivation of a plasmid free strain.** *Biotechnol Bioeng* 2012, **109**:1518–1527.
- Popov YV, Uskach YL, Ledenev SM, Kroman DA, Pavlova VA: **Synthesis of a sulfur-containing additive to transmission lubricants based on the C₂₀–C₂₆ fraction of α -olefins.** *Russ J Appl Chem* 2013, **86**:447–449.
- Kioupis LI, Maginn EJ: **Molecular simulation of poly- α -olefin synthetic lubricants: impact of molecular architecture on performance properties.** *J Phys Chem B* 1999, **103**:10781–10790.
- Coon MJ: **Cytochrome P450: nature's most versatile biological catalyst.** *Annu Rev Pharmacol Toxicol* 2005, **45**:1–25.
- Munro AW, Girvan HM, McLean KJ: **Variations on a (t)heme-novel mechanisms, redox partners and catalytic functions in the cytochrome P450 superfamily.** *Nat Prod Rep* 2007, **24**:585–609.
- Poulos TL, Johnson EF: **Cytochrome P450: Structure, Mechanism, and Biochemistry.** 3rd edition. New York: Kluwer Academic/Plenum Publishers; 2005.
- Matsunaga I, Sumimoto T, Ueda A, Kusunose E, Ichihara K: **Fatty acid-specific, regioselective, and stereospecific hydroxylation by cytochrome P450 (CYP152B1) from *Sphingomonas paucimobilis*: substrate structure required for α -hydroxylation.** *Lipids* 2000, **35**:365–371.
- Matsunaga I, Ueda A, Fujiwara N, Sumimoto T, Ichihara K: **Characterization of the *ybdT* gene product of *Bacillus subtilis*: novel fatty acid β -hydroxylating cytochrome P450.** *Lipids* 1999, **34**:841–846.
- Joo H, Lin Z, Arnold FH: **Laboratory evolution of peroxide-mediated cytochrome P450 hydroxylation.** *Nature* 1999, **399**:670–673.
- Cirino PC, Arnold FH: **A self-sufficient peroxide-driven hydroxylation biocatalyst.** *Angew Chem Intl Ed* 2003, **42**:3299–3301.
- Andre C, Kim SW, Yu X-H, Shanklin J: **Fusing catalase to an alkane-producing enzyme maintains enzymatic activity by converting the inhibitory byproduct H₂O₂ to the cosubstrate O₂.** *Proc Natl Acad Sci USA* 2013, **110**:3191–3196.
- Roberts GA, Grogan G, Greter A, Flitsch SL, Turner NJ: **Identification of a new class of cytochrome P450 from a *Rhodococcus* sp.** *J Bacteriol* 2002, **184**:3898–3908.
- Lee DS, Yamada A, Sugimoto H, Matsunaga I, Ogura H, Ichihara K, Adachi S, Park SY, Shiro Y: **Substrate recognition and molecular mechanism of fatty acid hydroxylation by cytochrome P450 from *Bacillus subtilis*.** Crystallographic, spectroscopic, and mutational studies. *J Biol Chem* 2003, **278**:9761–9767.
- Shoji O, Fujishiro T, Nakajima H, Kim M, Nagano S, Shiro Y, Watanabe Y: **Hydrogen peroxide dependent monooxygenations by tricking the substrate recognition of cytochrome P450_{BSP}.** *Angew Chem Intl Ed* 2007, **46**:3656–3659.
- Fujishiro T, Shoji O, Nagano S, Sugimoto H, Shiro Y, Watanabe Y: **Crystal structure of H₂O₂-dependent cytochrome P450_{SPa} with its bound fatty acid substrate: insight into the regioselective hydroxylation of fatty acids at a position.** *J Biol Chem* 2011, **286**:29941–29950.
- Munro AW, Girvan HM, Mason AE, Dunford AJ, McLean KJ: **What makes a P450 tick?** *Trends Biochem Sci* 2013, **38**:140–150.
- Hrycay EG, Bandiera SM: **The monooxygenase, peroxidase, and peroxygenase properties of cytochrome P450.** *Arch Biochem Biophys* 2012, **522**:71–89.
- Matsunaga I, Sumimoto T, Ayata M, Ogura H: **Functional modulation of a peroxygenase cytochrome P450: novel insight into the mechanisms of peroxygenase and peroxidase enzymes.** *FEBS Lett* 2002, **528**:90–94.
- Schlichting I, Berendzen J, Chu K, Stock AM, Maves SA, Benson DE, Sweet RM, Ringe D, Petsko GA, Sligar SG: **The catalytic pathway of cytochrome P450cam at atomic resolution.** *Science* 2000, **287**:1615–1622.
- Girhard M, Schuster S, Dietrich M, Duerre P, Urlacher VB: **Cytochrome P450 monooxygenase from *Clostridium acetobutylicum*: a new α -fatty acid hydroxylase.** *Biochem Biophys Res Comm* 2007, **362**:114–119.
- Netto LE, Stadtman ER: **The iron-catalyzed oxidation of dithiothreitol is a biphasic process: hydrogen peroxide is involved in the initiation of a free radical chain of reactions.** *Arch Biochem Biophys* 1996, **333**:233–242.
- Lu X, Vora H, Khosla C: **Overproduction of free fatty acids in *E. coli*: implications for biodiesel production.** *Metab Eng* 2008, **10**:333–339.
- Seaver LC, Imlay JA: **Hydrogen peroxide fluxes and compartmentalization inside growing *Escherichia coli*.** *J Bacteriol* 2001, **183**:7182–7189.

45. Bennett BD, Kimball EH, Gao M, Osterhout R, Van Dien SJ, Rabinowitz JD: **Absolute metabolite concentrations and implied enzyme active site occupancy in *Escherichia coli*.** *Nat Chem Biol* 2009, **5**:593–599.
46. Guengerich FP: **Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity.** *Chem Res Toxicol* 2001, **14**:611–650.
47. Guengerich FP, Munro AW: **Unusual cytochrome P450 enzymes and reactions.** *J Biol Chem* 2013, **288**:17065–17073.
48. Fujii T, Nakamura K, Shibuya K, Tanase S, Gotoh O, Ogawa T, Fukuka H: **Structural characterization of the gene and corresponding cDNA for the cytochrome P450_{rm} from *Rhodotorula minuta* which catalyzes formation of isobutene and 4-hydroxylation of benzoate.** *Mol Gen Genet* 1997, **256**:115–120.
49. Fasan R, Crook NC, Peters MW, Meinhold P, Buelter T, Landwehr M, Cirino PC, Arnold FH: **Improved product-per-glucose yields in P450-dependent propane biotransformations using engineered *Escherichia coli*.** *Biotechnol Bioeng* 2011, **108**:500–510.
50. Wei X-X, Chen G-Q: **Applications of the Vhb gene *vgb* for improved microbial fermentation processes.** *Methods Enzymol* 2008, **436**:273–287.
51. Bai L, Li P, Zhang H, Duan Y, Lin Z: **Semi-rational engineering of cytochrome P450_{sca-2} in a hybrid system for enhanced catalytic activity: insights into the important role of electron transfer.** *Biotechnol Bioeng* 2013, **110**:2815–2835.
52. Yu X, Liu T, Zhu F, Khosla C: ***In vitro* reconstitution and steady-state analysis of the fatty acid synthase from *Escherichia coli*.** *Proc Natl Acad Sci USA* 2011, **108**:18643–18648.
53. Liu T, Vora H, Khosla C: **Quantitative analysis and engineering of fatty acid biosynthesis in *E. coli*.** *Metab Eng* 2010, **12**:378–386.
54. Sullivan KH, Hegeman GD, Cordes EH: **Alteration of the fatty acid composition of *Escherichia coli* by growth in the presence of normal alcohols.** *J Bacteriol* 1979, **138**:133–138.
55. Marr AG, Ingraham JL: **Effect of temperature on the composition of fatty acids in *Escherichia coli*.** *J Bacteriol* 1962, **84**:1260–1267.
56. Akhtar MK, Turner NJ, Jones PR: **Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities.** *Proc Natl Acad Sci USA* 2013, **110**:87–92.
57. Gogerty DS, Bobik TA: **Formation of isobutene from 3-hydroxy-3-methylbutyrate by diphosphomevalonate decarboxylase.** *Appl Environ Microbiol* 2010, **76**:8004–8010.
58. Heckman KL, Pease LR: **Gene splicing and mutagenesis by PCR-driven overlap extension.** *Nat Protoc* 2007, **2**:924–932.
59. Li S, Podust LM, Sherman DH: **Engineering and analysis of a self-sufficient biosynthetic cytochrome P450 PikC fused to the RhFRED reductase domain.** *J Am Chem Soc* 2007, **129**:12940–12941.
60. Li S, Chaulagain MR, Knauff AR, Podust LM, Montgomery J, Sherman DH: **Selective oxidation of carbolide C–H bonds by an engineered macrolide P450 mono-oxygenase.** *Proc Natl Acad Sci USA* 2009, **106**:18463–18468.
61. Anzai Y, Li S, Chaulagain MR, Kinoshita K, Kato F, Montgomery J, Sherman DH: **Functional analysis of MycCl and MycG, cytochrome P450 enzymes involved in biosynthesis of mycinamicin macrolide antibiotics.** *Chem Biol* 2008, **15**:950–959.
62. Omura T, Sato R: **The carbon monoxide-binding pigment of liver microsomes. II. Solubilization, purification, and properties.** *J Biol Chem* 1964, **239**:2379–2385.
63. Fujii K, Galivan JH, Huennekens FM: **Activation of methionine synthase: further characterization of flavoprotein system.** *Arch Biochem Biophys* 1977, **178**:662–670.
64. Jenkins CM, Waterman MR: **NADPH-flavodoxin reductase and flavodoxin from *Escherichia coli*: characteristics as a soluble microsomal P450 reductase.** *Biochemistry* 1998, **37**:6106–6113.
65. Guan W, Zhao H, Lu X, Wang C, Yang M, Bai F: **Quantitative analysis of fatty-acid-based biofuels produced by wild-type and genetically engineered cyanobacteria by gas chromatography–mass spectrometry.** *J Chromatogr A* 2011, **1218**:8289–8293.
66. Bauer S, Schulte E, Thier H-P: **Composition of the surface wax from tomatoes. I. Identification of the components by GC/MS.** *Eur Food Res Technol* 2009, **219**:223–228.

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