

Discovery of a Family of Desaturase-Like Enzymes for 1-Alkene Biosynthesis

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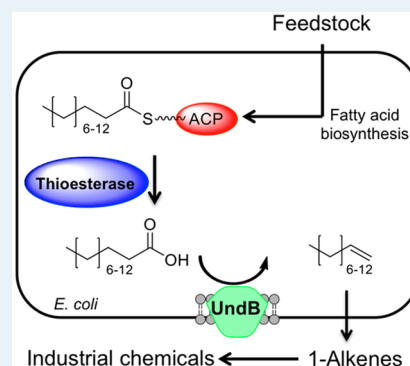
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S Supporting Information

ABSTRACT: 1-Alkenes are important platform chemicals that are almost exclusively produced from fossil hydrocarbons. Bioproduction of 1-alkenes can mitigate our dependence on declining petrochemical resources, thereby representing an important step in the field of green chemistry. Here, we report the discovery of a new family of membrane-bound desaturase-like enzymes that convert medium-chain fatty acids (10–16 carbons) into the corresponding 1-alkenes through oxidative decarboxylation. We further show that these desaturase-like enzymes could be efficient in transforming lauric acid to 1-undecene in *E. coli* compared to the existing 1-alkene biosynthetic enzymes. This work expands the enzyme inventory for the transformation of fatty acid precursors to hydrocarbons and promotes the industrial production of medium-chain 1-alkenes through microbial fermentation.

KEYWORDS: 1-alkene, 1-undecene, desaturase, biocatalysis, biosynthesis



Terminal alkene hydrocarbons are important platform chemicals with an industrial production of well over 100 000 t per year.¹ They are overwhelmingly used as essential precursors for lubricants, detergents, greases, pesticides, and cosmetic products.² The unique utility of these hydrocarbons arises from the terminal double bond, which allows for versatile derivatization and facile polymerization when compared to saturated alkanes or fatty alcohols. Currently, 1-alkenes are exclusively derived from petroleum, which is nonrenewable and energetically costly. Given the tremendous importance and industrial value of 1-alkenes, the foreseeable depletion of fossil resources, and surging environmental concerns, there is an urgent demand for alternative supply routes for 1-alkenes.

The importance of the terminal olefin for the chemical industry is contrasted by the fact that nature does not produce significant amounts of 1-alkenes. To date, only trace amounts of 1-alkenes have been found to be produced by selected organisms, presumably assisting the survival of the producing hosts.³ The low natural occurrence of 1-alkenes has led to an apparent knowledge gap in biocatalysis for terminal olefin synthesis. It was not until recently that several enzymatic reactions for the synthesis of terminal alkenes were discovered (Figure 1). One example is OleTJE, a cytochrome P450 fatty acid peroxxygenase/decarboxylase that catalyzes the conversion of long-chain fatty acids into 1-alkenes in *Jeotgalicoccus* sp. 8456.⁴ Another example involves a multidomain polyketide synthase, which synthesizes very long-chain 1-alkenes via an elongation/sulfonation/decarboxylation mechanism in cyanobacteria.⁵ We reported the discovery of a family of oxidases that

are responsible for the biosynthesis of 1-undecene and are conserved in *Pseudomonas*.⁶ The representative enzyme, UndA, is able to convert medium-chain fatty acids (C10–C14) into their corresponding “C-1” 1-alkenes using an oxygen-activating, nonheme iron-dependent mechanism. Being the only natural catalysts for medium-chain 1-alkene synthesis, UndA and its homologues open up a new perspective for the bioproduction of volatile 1-alkenes.¹ However, all of the reported 1-alkene biosynthetic machinery seems to have low catalytic efficiency, and the reported titers of 1-alkenes in *E. coli* are rather far from synthetic applications. The scarce inventory of terminal olefin-producing enzymes calls for new enzyme discovery to advance the industrial synthesis of biobased olefins from renewable resources.

During our previous work with UndA, we observed that the titers of 1-undecene varied within 2 orders of magnitude (1–100 $\mu\text{g/L}$) in cultures of different *Pseudomonas* species, whereas the heterologous expression of homologous genes to *unda* from these species led to the production of 1-undecene at similar titers (3–6 mg/L) in *E. coli*.⁶ Although different levels of gene expression and/or substrate supply in *Pseudomonas* might provide a partial explanation for the difference in observed titers, we hypothesized that there could be additional biosynthetic pathways for 1-undecene in some, but not all, *Pseudomonas* species. We then rescreened the genomic library

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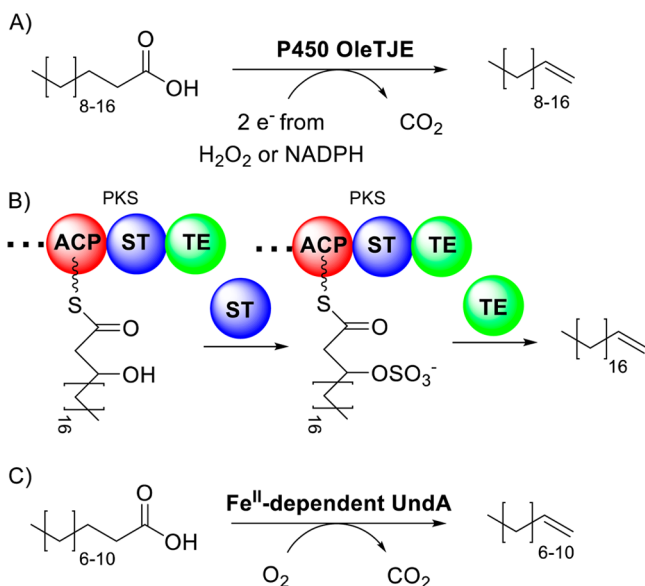


Figure 1. Examples of known enzymatic reactions for 1-alkene biosynthesis. (A) OleTJE, a cytochrome P450 fatty acid peroxxygenase/decarboxylase catalyzes the conversion of long-chain fatty acids into 1-alkenes. (B) A multidomain polyketide synthase (PKS) synthesizes very long-chain 1-alkenes via an elongation/sulfonation/decarboxylation mechanism. ACP: acyl carrier protein, ST: sulfotransferase, TE: thioesterase. (C) UndA converts medium-chain fatty acids into their corresponding “C-1” 1-alkenes using an oxygen-activating, nonheme iron-dependent mechanism.

of *Pseudomonas fluorescens* Pf-5, one of the better 1-undecene producers, through heterologous expression in *E. coli* with the feeding of free lauric acid (LA) (Figure 2). We expected that

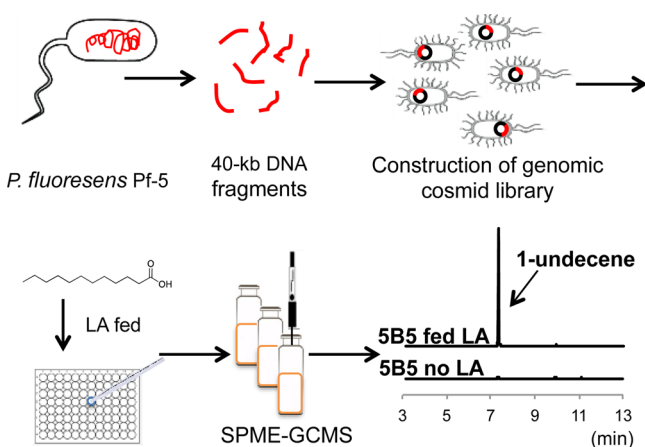


Figure 2. Identification of 1-undecene biosynthetic gene(s) by heterologous expression of the *Pseudomonas* genomic library in *E. coli* with LA feeding, followed by 1-undecene accumulation analysis using headspace solid phase microextraction (SPME)–gas chromatography mass spectrometry (GCMS) detection method.

LA feeding could potentially boost the production of 1-undecene and increase the sensitivity of screening. The subsequent 1-undecene production analysis yielded one additional clone, 5B5, which led to the production of 1-undecene at a titer of 100 $\mu\text{g/L}$ when expressed in *E. coli*. It is notable that the production of 1-undecene was barely noticeable without the feeding of LA (Figure 2).

DNA sequencing analysis revealed that the genomic insert in 5B5 does not encode UndA, indicating an alternative mechanism for 1-undecene synthesis in *E. coli*. To identify the specific gene(s) responsible for 1-undecene production, we combined the approach of trimming the genomic insert in 5B5 using restriction enzymes and overexpressing individual candidate genes in *E. coli* (Figure S1). We found that one single gene *PFL_0203*, designated as *undB*, is sufficient and necessary for 1-undecene production in *E. coli*. Indeed, the *E. coli* strain UND001, in which *undB* was cloned into a pET vector, produced 1-undecene at a titer of 10 mg/L when supplemented with LA.

Sequence homology analysis suggested that UndB belongs to a membrane-bound fatty acid desaturase family that typically removes two hydrogen atoms from fatty acids to introduce a double bond. Consistent with all other membrane-bound desaturases,⁷ UndB is predicted to contain transmembrane domains and three conserved His-boxes, H₈₉DLIH₉₃, H₁₂₈(L/F)(N/H)H₁₃₁H₁₃₂, and H₂₉₇(G/S/A)IH₃₀₀H₃₀₁, which presumably include the ligands for a di-iron cluster at the catalytic site of the enzyme (Figure S2). UndB is thus proposed to catalyze the oxidative decarboxylation of LA to form 1-undecene through a mechanism similar to the fatty acid desaturation (Figure S3).^{7a,8} To confirm that LA is the 1-undecene biosynthetic precursor and the terminal carboxylic acid moiety is removed during 1-undecene formation, we fed [12-¹³C]LA and [1-¹³C]LA to the culture of UND001. As expected, [11-¹³C]undecene and [U-¹²C11]undecene were produced, respectively (Figure S4). In addition, when [α,α -D2]LA was fed to the culture of UND001, 1-undecene retaining both deuterium atoms was produced (Figure S4), suggesting LA is likely activated through β -hydrogen abstraction by UndB, similar to UndA (Figure S3).

It is notable that desaturases typically accept CoA, acyl carrier protein (ACP), or glycerolipid substrates, and the recognition of a free fatty acid is rare. To probe if LA is a direct substrate for UndB, we coexpressed *UcFatB2*, which encodes a thioesterase that specifically releases the lauryl chain from the fatty acid biosynthetic pathway to form LA in *E. coli*.⁹ After optimization of the coexpression conditions of *undB* and *UcFatB2* in *E. coli*, the best strain UND003 produced 1-undecene at a titer of ~ 22 mg/L without LA feeding. This titer of 1-undecene is over 100-fold higher than the control strain without *UcFatB2*, suggesting that UndB likely directly functions on free fatty acids to yield 1-alkenes (Figure 3 and S5). These in vivo data are consistent with our results of reconstituted UndB activity in vitro. In particular, we purified the UndB-containing membrane fraction of UND001 for use in biochemical assays (Figure S6). To circumvent the problem of 1-undecene background in the membrane fraction, we used labeled LA including [α,α -D2]LA and [12-¹³C]LA as substrates. Conversion of labeled LA to labeled 1-undecene was clearly observed (Figure S7), further indicating that free fatty acid is the direct substrate for UndB.

In silico analysis revealed that homologues of UndB are present in only a few *Pseudomonas* and related species and are not as widely spread as UndA (Figure S8). For example, many large *Pseudomonas* subspecies, such as *P. aeruginosa* and *P. putida*, do not have UndB homologues. This observation is consistent with our hypothesis that this alternative 1-alkene biosynthetic pathway only exists in selected *Pseudomonas* strains. To further probe the function of UndB homologues in the biosynthesis of 1-undecene, we coexpressed two

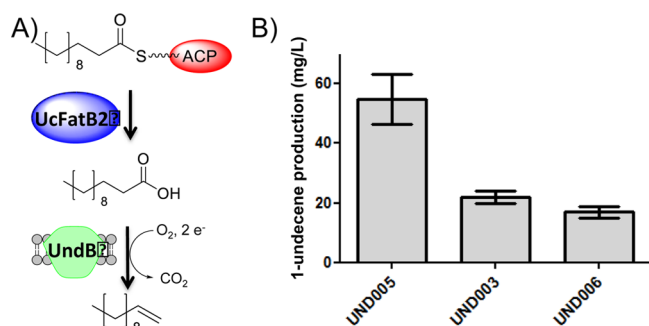


Figure 3. 1-Undecene production in the engineered *E. coli* strains. (A) Biosynthetic pathway of 1-undecene production in engineered *E. coli* strains. (B) Comparison of 1-undecene production in *E. coli* strains that express *undB* (UND003), *Pmen_4370* (UND005), and *ABAYE3084* (UND006), respectively. Error bars represent standard deviations from at least three independently performed experiments.

additional *undB* homologues with *UcFatB2* in *E. coli*. *Pmen_4370* from *Pseudomonas mendocina* *ymp* and *ABAYE3084* from *Acinetobacter baumannii* AYE were selected, which exhibit identity/similarity of 76%/85% and 59%/73% to *UndB*, respectively. Overexpression of these gene candidates in *E. coli* conferred the ability to produce 1-undecene, confirming the proposed function of these genes (Figure 3B). It is notable that the strain UND005 with coexpression of *Pmen_4370* and *UcFatB2* produced 1-undecene at a titer of ~55 mg/L, indicating that this homologue has higher 1-alkene production efficiency than *UndB* in *E. coli*. This result further suggested that screening of additional *UndB* homologues would likely yield catalysts that have improved 1-alkene synthesis efficiency in desired production hosts.

To date, three different types of enzymes have been discovered that are capable of converting LA to 1-undecene. These include the cytochrome P450 enzyme *OleTJE*, the nonheme iron-dependent *UndA* family, and the *UndB* family of membrane-bound desaturase-like enzymes. To directly compare the in vivo efficiency of these enzymes in *E. coli*, we replaced *Pmen_4370* in UND005 with *OleTJE* and *Pput_3952* (an *undA* homologue)⁶ to yield strains UND007 and UND008, respectively. The subsequent fermentation using the same conditions demonstrated that UND005 produced at least 3-fold more 1-undecene than the other two strains (Figure 4A). To exclude the possibility that the decreased titers of 1-undecene in

UND007 and UND008 were related to the decreased production of LA substrates, we further examined the levels of extracellular free LA in these 1-undecene producing cultures. Significant quantities of LA were detected in the fermentation media of UND007 and UND008 but not UND005 (Figure 4B), suggesting that upon generation of LA by the thioesterase in *E. coli*, *OleTJE* or *UndA* was not able to transform intracellular free LA in timely fashion and some of the LA were exported and lost to the fermentation media. These results demonstrated the membrane-bound desaturase-like enzyme was the most efficient catalyst among the three enzymes for producing 1-undecene in *E. coli*, and the calculated conversion of LA reached >95%.

To further expand the potential of *UndB* homologues as biocatalysts to produce 1-alkenes with altered chain length, we examined the chain length specificity of *UndB* by feeding the cultures of UND001 fatty acids ranging from C₆–C₁₈. GC-MS analysis of each culture showed the production of the corresponding “C-1” 1-alkenes, albeit at varied titers (Figure 5A and S9). These data showed that *UndB* prefers fatty acids of C₁₀–C₁₄, with modest conversion of palmitic acid (C₁₆) and low conversion of longer or shorter-chain fatty acids. This result is consistent with the in vivo 1-alkene production profile of *Pmen_4370* when a thioesterase of different chain length specificity was used (Figure 5B). Particularly, when *UcFatB2* in UND005 was replaced by a gene encoding “*TesA*(L109P)”,¹⁰ a C₁₄-specific thioesterase, the predominant product was switched from 1-undecene to 1-tridecene from the newly generated strain, UND009 (Figure 5B). These results demonstrated that in addition to *UndA*, *UndB* represents the second family of natural catalysts discovered thus far that specifically synthesize medium-chain 1-alkenes.

In summary, we discovered a new family of desaturase-like enzymes that convert medium-chain fatty acids into the corresponding terminal olefins through oxidative decarboxylation. Compared to other 1-alkene producing enzymes, these membrane-bound desaturase-like enzymes are the most efficient biocatalysts hitherto for medium-chain 1-alkenes, especially 1-undecene production in *E. coli*. A radical mechanism started with β -hydrogen abstraction of fatty acids was proposed for *UndB*; however, the notorious difficulty in working with membrane-bound desaturases in vitro limits the mechanistic understanding of this family of enzymes. Nonetheless, membrane-bound oxidative enzymes can be efficiently

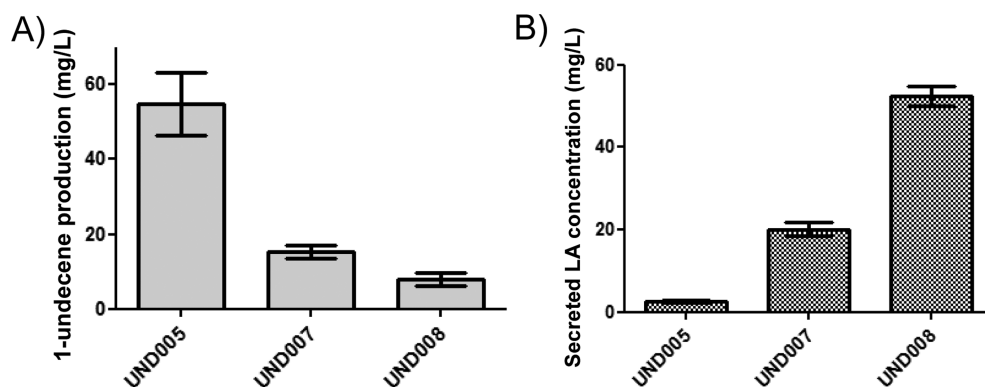


Figure 4. Comparison of secreted 1-undecene and LA levels by different *E. coli* strains. (A) Comparison of 1-undecene production in *E. coli* strains expressing *undB* (UND005), *OleTJE* (UND007), and *Pput_3952* (UND008), respectively. (B) Comparison of secreted free LA levels in the corresponding 1-undecene producing *E. coli* strains. Error bars represent standard deviations from at least three independently performed experiments.

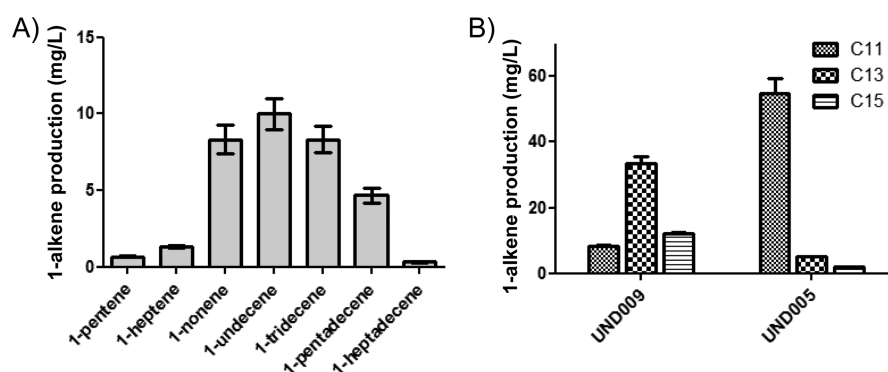


Figure 5. 1-Alkene production by the engineered *E. coli* strains. (A) 1-Alkene production by feeding UND001 with various fatty acids of different chain lengths. (B) 1-Alkene distributions by *E. coli* strains that coexpress *Pmen_4370/TesA(L109P)* (UND009, left panel) and *Pmen_4370/UcFatB2* (UND005, right panel), respectively. Error bars represent standard deviations from at least three independently performed experiments.

developed for whole-cell biotransformations.¹¹ Our findings expand the scarce enzyme inventory for terminal olefin biosynthesis and promote the establishment of a bioprocess to produce medium-chain 1-alkenes from renewable resources.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acscatal.5b01842.

Details of experimental procedures, primers, GC-MS analysis, and other supplemental data (PDF)

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Notes

The authors declare no competing financial interest.

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