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Enabling the synthesis of medium chain alkanes and 1-alkenes in yeast

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ABSTRACT

Microbial synthesis of medium chain aliphatic hydrocarbons, attractive drop-in molecules to gasoline and jet fuels, is a promising way to reduce our reliance on petroleum-based fuels. In this study, we enabled the synthesis of straight chain hydrocarbons (C7–C13) by yeast *Saccharomyces cerevisiae* through engineering fatty acid synthases to control the chain length of fatty acids and introducing heterologous pathways for alkane or 1-alkene synthesis. We carried out enzyme engineering/screening of the fatty aldehyde deformylating oxygenase (ADO), and compartmentalization of the alkane biosynthesis pathway into peroxisomes to improve alkane production. The two-step synthesis of alkanes was found to be inefficient due to the formation of alcohols derived from aldehyde intermediates. Alternatively, the drain of aldehyde intermediates could be circumvented by introducing a one-step decarboxylation of fatty acids to 1-alkenes, which could be synthesized at a level of 3 mg/L, 25-fold higher than that of alkanes produced *via* aldehydes.

1. Introduction

Medium chain aliphatic hydrocarbons (C7–C13) are the predominant components in petroleum-based gasoline or jet fuels (Edwards, 2003; Sheppard et al., 2016), and also serve as solvents and chemicals (Choi and Lee, 2013; Kourist, 2015). A variety of enzymes committed to the formation of hydrocarbons *via* the deoxygenation of fatty acids (Kourist, 2015; Rude et al., 2011; Rui et al., 2015, 2014) or fatty aldehydes (Aarts et al., 1995; Marsh and Waugh, 2013; Qiu et al., 2012; Schirmer et al., 2010) have been discovered in nature (Fig. 1). In addition, the production of straight chain alkanes or 1-alkenes *via* biocatalysis (Amaya et al., 2016; Dennig et al., 2015; Foo et al., 2017; Liu et al., 2014; Yan et al., 2015; Zachos et al., 2015; Zhang et al., 2013) or heterologous biosynthesis (Akhtar et al., 2013; Bernard et al., 2012; Buijs et al., 2015; Cao et al., 2016; Chen et al., 2015; Choi and Lee, 2013; Coursolle et al., 2015; Foo et al., 2017; Harger et al., 2013; Howard et al., 2013; Kallio et al., 2014; Liu et al., 2014; Schirmer et al., 2010; Sheppard et al., 2016; Song et al., 2016; Xu et al., 2016; Yan et al., 2016; Zhou et al., 2016a, 2016b) has been implemented. Yeast, an important industrial workhorse (Becker and Wittmann, 2015; Nielsen, 2015), is considered to be very suitable for the production of these fuel molecules (Hong and Nielsen, 2012). However, *in vivo* synthesis of medium chain aliphatic hydrocarbons in yeast is not realized yet. This is mostly because of the scarcity of medium chain fatty acid (MCFA) precursors in native yeast cells. Recently, we have modified fungal type I fatty acid synthases (FASs) by inserting a heterologous thioesterase to release MCFAs and introducing mutations into the ketoacyl synthase domain (G1250S and M1251W in Fas2) to restrict the elongation of fatty acids (Gajewski et al., 2017a; Zhu et al., 2017). These modifications in FASs have allowed yeast to produce MCFAs. Here we further enabled the *in vivo* synthesis of medium chain alkanes and 1-alkenes in yeast by exploring the activities of hydrocarbon-forming enzymes towards medium chain substrates, and blocking the formation of by-products.

Abbreviations: MCFA, medium chain fatty acid; FAS, fatty acid synthase; Headspace/SPME/GC/MS, Headspace/solid-phase microextraction/gas chromatography/mass spectrometry; ADO, aldehyde deformylating oxygenase; CAR, carboxylic acid reductase

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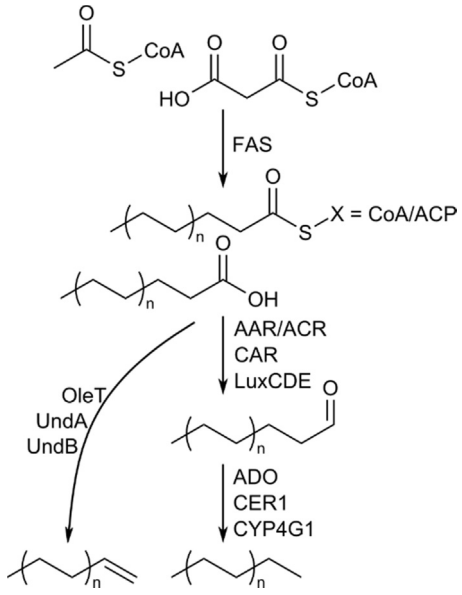

Fig. 1. Biosynthetic pathways of straight chain aliphatic hydrocarbons. ACP, acyl carrier protein; CoA, coenzyme A; FAS, fatty acid synthase; AAR, acyl-ACP reductase; ACR, acyl-CoA reductase; CAR, carboxylic acid reductase; LuxCDE, fatty acid reductase complex components LuxC (reductase), LuxD (synthetase) and LuxE (transferase); ADO, cyanobacterial fatty aldehyde deformylating oxygenase; CER1, plant membrane-bound decarbonylase; CYP4G1, insect-specific P450 oxidative decarbonylase of the CYP4G family from *Drosophila*; OleT, P450 fatty acid decarboxylase; UndA, non-heme oxidase/decarboxylase; UndB, membrane-bound desaturase-like decarboxylase.

2. Materials and methods

2.1. Plasmids, strains and culture conditions

Strains and plasmids used in this study were listed in Table S1 and Table S2, respectively. *E. coli* DH5α was used for plasmid amplification. If not specified, *E. coli* cells were cultivated in Luria-Bertani (LB) medium at 37 °C and 200 rpm. 80 mg/L of ampicillin was supplemented for plasmid selection. Transformation of *E. coli* was according to a previously described protocol (Inoue et al., 1990). All *S. cerevisiae* strains used in this study were derived from CEN.PK113-11C (*MATa SUC2 MAL2-8c his3Δ1 ura3-52*). "YPD" medium (10 g/L yeast extract, 20 g/L peptone, and 20 g/L glucose) was used for regular culture of yeasts. "YPD + G418″ medium containing 200 mg/L G418 (Formedium, Hunstanton, UK) was used for selection of transformants with a *kanMX* cassette. "SC-URA" medium containing 20 g/L glucose, 6.7 g/L yeast nitrogen base without amino acids (YNB, Formedium, Hunstanton, UK), and 0.77 g/L complete supplement mixture without uracil (CSM-URA, Formedium, Hunstanton, UK) was used for selection of transformants prototrophic to uracil. "SC + 5FOA" medium containing 6.7 g/L YNB, 0.79 g/L complete supplement mixture (CSM, Formedium, Hunstanton, UK) and 0.8 g/L 5-fluoroorotic acid (5-FOA, Sigma-Aldrich) was used for recycling of *URA3* marker. 20 g/L agar (Merck Millipore) was added to make solid media. The recipe of Delft medium was described before (Jensen et al., 2014). 100 mg/L histidine and/or 100 mg/L uracil were supplemented as needed. If not specified, 20 g/L glucose was used as carbon source. Yeast cells were cultivated at 30 °C and 200 rpm in liquid media. A LiAc/SS carrier DNA/PEG method (Gietz and Schiestl, 2007) was used for yeast transformation. Cell density (OD at 600 nm) was measured by GENESYS 20 spectrophotometer (Thermo Fisher Scientific).

2.2. Plasmid and strain construction

Codon-optimized genes were synthesized by GenScript (Piscataway, NJ, USA) and listed in Table S3. Primers used in this study (Table S4) were synthesized by Sigma-Aldrich or Eurofins Genomics. PrimeStar DNA polymerase (Takara Bio), Zymoprep Yeast Plasmid Miniprep II kit (Zymo Research Corp), Restriction enzymes, DreamTaq DNA Polymerase, DNA gel purification and plasmid extraction kits (Thermo Fisher Scientific), and Gibson Assembly Master Mix (New England BioLabs) were used according to the manufacturers' instructions. DNA sequencing was performed by Eurofins Genomics.

ADO and UndA-like genes were inserted into vectors pZWM1 (*TEF1* promoter and *ADH1* terminator) or pZWM2 (enhanced *TDH3* promoter and *CYC1* terminator) by using the Gibson assembly cloning kit and primers 1–36. Internal primer pairs (37–52 in Table S4) were used for introducing site-directed mutations into ADO genes. All plasmids were verified by sequencing.

The DNA assembler method (Shao et al., 2009; Zhou et al., 2012) was used for the construction of pAlkane261 and pAlkane262 plasmids, and primers 53–65 were used to amplify DNA fragments from pAlkane26 (Zhou et al., 2016a) or pZWM2-TeADO. A yeast 2μ vector pYX212 containing a *URA3* selection marker was used and linearized by digestion with restriction enzymes SphI and EcoRI. The PCR fragments accompanied with the linearized vector were transformed into *S. cerevisiae* YJZ02 and selected on "SC-URA" plates. Plasmids extracted from transformants by the Zymoprep Yeast Plasmid Miniprep II kit were transformed into *E. coli* DH5α. The plasmids extracted from *E. coli* were verified by restriction digestion and sequencing.

CRISPR/Cas9-mediated genome engineering was implemented by co-transformation of a repair fragment, and an all-in-one plasmid pECAS9-gRNA expressing high-fidelity Cas9 nuclease (eCas9) (Slaymaker et al., 2016) and guide RNA. The high-fidelity version of Cas9 was obtained by using primers 66–73 to introduce K848A, K1003A, and R1060A mutations into the TEF1p-Cas9-CYC1t cassette from p414-TEF1p-Cas9-CYC1t (DiCarlo et al., 2013). The guide RNA expression cassette (targeting *HFD1* gene) was obtained by using primers 74–77 and pROS10 (Mans et al., 2015) as DNA template. The 2μ vector backbone was amplified from pYX212 by using primers 78–79, and the *kanMX* and *KlURA3* selection markers were amplified from pUG6 (Gueldener et al., 2002) and pWJ1042 (Reid et al., 2002), respectively, by using primers 80–83. The pECAS9-gRNA-KlURA3-tHFD1 and pECAS9-gRNA-kanMX-tHFD1 plasmids were generated by Gibson assembly cloning. The 100 bp repair fragment bridging a 50 bp upstream sequence and a 50 bp downstream sequence of the *HFD1* open reading frame was generated by PCR amplification with primers 84–85 which had a 20-bp overlap. For the construction of YJZ03 (*Δpox1* & *Δhfd1*) isogenic strain, 1 μg of pECAS9-gRNA-kanMX-tHFD1 and 200 ng of the repair fragment were transformed into YJZ02 (*Δpox1*), and "YPD + G418″ plates were used for selection of transformants. While for the deletion of *hfd1* in ZW207 strain (*kanMX*$^+$), 1 μg of pECAS9-gRNA-KlURA3-tHFD1 and 200 ng of the repair fragment were used for transformation, and "SC-URA" plates were used for selection of transformants. Primers 86–87 were used for PCR verification of *hfd1* deletion. After confirmation of the deletion, the strains were cultivated in non-selective medium (YPD) to remove the corresponding plasmids.

ZW540 strain used for *in vivo* evaluation of ADO enzymes was constructed as described below. The *MmCAR*, *npgA*, *EcFd,* and *EcFNR* genes expressed in ZW540 were described previously (Zhou et al., 2016b). Primers 88–105 were used to generate cassettes for the integration of GAL7p-MmCAR-ADH1t and GAL3p-npgA-FBA1t into the *GAL1/10/7* locus. These cassettes were transformed into YJZ03 and selected on "SC-URA" plates. After colony PCR verification (Looke et al., 2011), the strain carrying correct integration cassettes was selected on "SC + 5FOA" plates to recycle the *URA3* marker. Similarly, primers 106–121 were used to generate cassettes for the further integration of GAL1p-EcFNR-CYC1t and GAL10p-EcFd-TDH2t into the *GAL80* locus, and the strain obtained after removal of *URA3* marker was designated as YJZ54. *ScFAS27* was integrated into YJZ54 as described before (Zhu et al., 2017) to generate strain ZW540.