Engineering *Yarrowia lipolytica* as a platform for synthesis of drop-in transportation fuels and oleochemicals

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Harnessing lipogenic pathways and rewiring acyl-CoA and acyl-ACP (acyl carrier protein) metabolism in *Yarrowia lipolytica* hold great potential for cost-efficient production of diesel, gasoline-like fuels, and oleochemicals. Here we assessed various pathway engineering strategies in *Y. lipolytica* toward developing a yeast biorefinery platform for sustainable production of fuel-like molecules and oleochemicals. Specifically, acyl-CoA/acyl-ACP processing enzymes were targeted to the cytoplasm, peroxisome, or endoplasmic reticulum to generate fatty acid ethyl esters and fatty alkanes with tailored chain length. Activation of endogenous free fatty acids and the subsequent reduction of fatty acyl-CoAs enabled the efficient synthesis of fatty alcohols. Engineering a hybrid fatty acid synthase shifted the free fatty acids to a medium chain-length scale. Manipulation of alternative cytosolic acetyl-CoA pathways partially decoupled lipogenesis from nitrogen starvation and unleashed the lipogenic potential of *Y. lipolytica*. Taken together, the strategies reported here represent promising steps to develop a yeast biorefinery platform that potentially upgrades low-value carbons to high-value fuels and oleochemicals in a sustainable and environmentally friendly manner.

metabolic engineering | synthetic biology | oleochemicals | advanced biofuels | oleaginous yeast

Engineering microbes for the production of fuels and green chemicals provides a promising path to reducing our dependence on fossil fuels and mitigating climate change concerns. Recently, fatty acid-based fuels have been receiving growing attention due to their unique advantages such as higher energy density, lower hygroscopicity, miscibility with diesel fuels, reduced purification costs, and compatibility with existing infrastructure (1). As such, extensive efforts have been made to engineer various microbes to produce free fatty acids (2, 3), fatty alcohols (4), fatty acid ethyl esters (FAEEs) (5–7), and alkanes (4, 8). Most of the work has involved engineering bacteria or baker's yeast, with varying outcomes to date.

Here we investigate *Yarrowia lipolytica* toward building a sustainable oleaginous yeast factory for production of diesel-like fuels and oleochemicals from carbohydrate resources. High acetyl-CoA flux and the oil sequestration mechanism have highlighted this yeast as a superior lipid biosynthetic factory (9). Prior work using flux push-and-pull strategies along with expression of D9 desaturase has led to the construction of efficient single-cell oil factories with high titer (>55 g/L) and yield (0.234 g lipids per g glucose) (10). These results suggest that oleaginous yeasts could be further engineered to biologically synthesize fuel-like molecules and oleochemicals, provided that we can rewire their acyl-CoA/acyl-ACP metabolism (Fig. 1*A* and *SI Appendix*, Fig. S1). However, compared with their prokaryotic counterparts, our ability to engineer eukaryotic lipid metabolism is not as developed, and progress to date has yielded only mg/L-level quantities of alkanes (4, 11) and FAEEs (4, 5). Efficient synthesis of fuel-like molecules and oleochemicals requires a more detailed understanding of the lipogenic mechanism and the associated biophysical processes in oleaginous yeast.

Results and Discussion

FAEE and Fatty Alkane Production in *Y. lipolytica*. Eukaryotes have evolved to partition specialized metabolic functions into distinct cellular compartments. The biogenesis of lipids, acyl-CoAs, and acyl-ACPs occurs in discrete subcellular compartments and often involves spatially separated enzymatic reactions (12, 13). For example, triacylglycerides (TAGs) are synthesized in the endoplasmic reticulum (ER) and further agglomerate into lipid bodies (14) (Fig. 1*B*). Free fatty acids and acyl-CoA intermediates are degraded into acetyl-CoAs via β-oxidation pathways in the peroxisome (15) (Fig. 1*B*). Acyl-CoAs formed in the cytoplasm are primarily retrieved by acyl-CoA–binding proteins (16) and translocated to either the ER for lipid biosynthesis or peroxisome for β-degradation. This compartmentalization makes the acyl-CoA and acyl-ACP intermediates inaccessible to the downstream engineered biocatalysts that are necessary to convert them to various fuels and oleochemicals. We thus investigated whether expression of enzymes in specific subcellular organelles (enzyme targeting) would be beneficial in diverting acyl-CoAs and acyl-ACPs toward the formation of downstream fuel molecules, namely FAEEs and fatty alkanes. We found that cytosolic expression of *Acinetobacter baylyi* ADP1 wax-ester synthase *Ab*AtfA (17) only results in marginal FAEEs (7.1 mg/L) (Fig. 2 *A* and *B*). Similarly, cytosolic coexpression of *A. baylyi* ADP1 fatty acyl-CoA reductase *Ab*ACR1 (18) and *Prochlorococcus marinus* aldehyde deformylating oxygenase *Pm*ADO (19) led to fatty alkane production of

Significance

Cost-efficient production of fuels and oleochemicals requires the host strain to be highly lipogenic and suitable for large-scale production. We demonstrate that *Yarrowia lipolytica* represents a promising biorefinery platform for sustainable production of drop-in transportation fuels and oleochemicals. Understanding the mechanistic details of the lipogenic phenotype, particularly the cellular compartmentalization of distinct metabolic pathways, fatty acid synthase structure, activating free fatty acids to acyl-CoAs, and decoupling nitrogen starvation from lipogenesis, allowed us to efficiently produce fatty acid ethyl esters, fatty alkanes, medium chain-length fatty acids, fatty alcohols, and triacylglycerides (TAGs). We envision that this report constitutes foundational work in developing an oleaginous yeast platform to upgrade low-value carbons to high-value fuels and oleochemicals in the foreseeable future.

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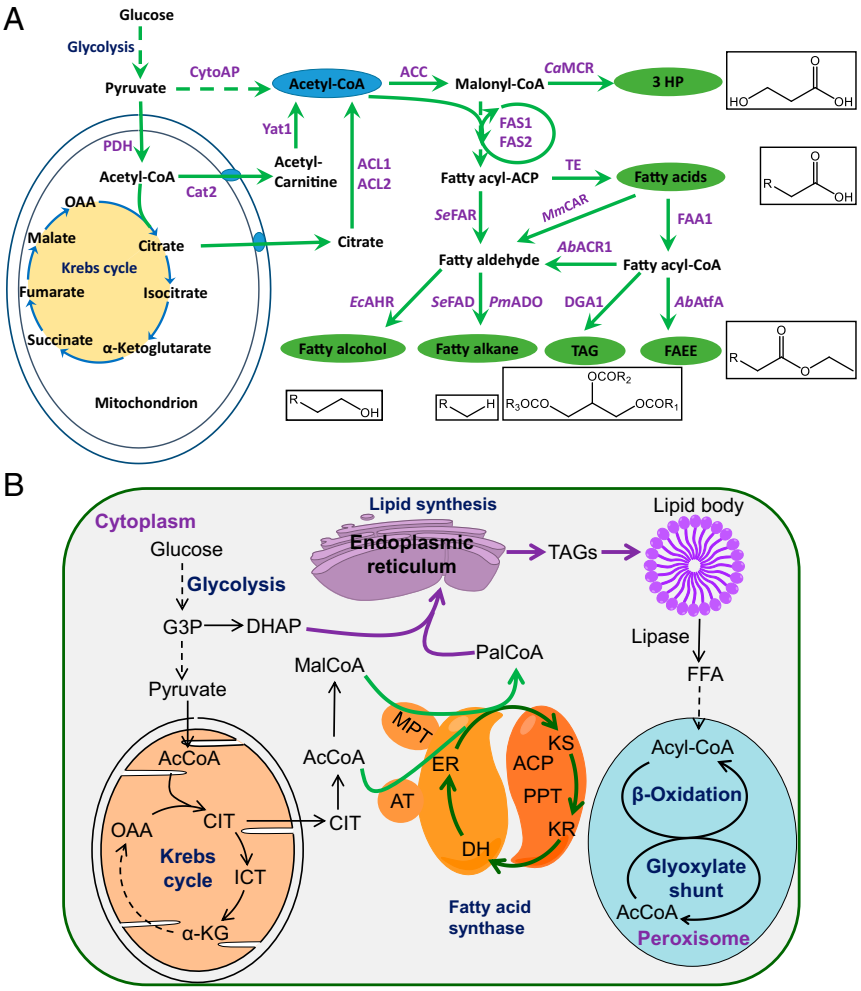

Fig. 1. Rewiring fatty acyl-CoA and fatty acyl-ACP metabolism for the production of fuels and oleochemicals. Green arrows indicate reactions engineered in this work. (*A*) Opportunity for engineering *Y. lipolytica* lipogenic chemistry to synthesize a broad range of fuels, oleochemicals, and green chemicals. *Ab*AtfA, wax-ester synthase from *A. baylyi* ADP1; ACC, acetyl-CoA carboxylase; *Ca*MCR, malonyl-CoA reductase from *Chloroflexus aurantiacus*; Cat2, carnitine acetyltransferase; CytoAP, cytosolic acetyl-CoA pathways; DGA1, diacylglycerol transferase; FAA1, fatty acyl-CoA synthase; 3 HP, 3-hydroxyl propionate; *Mm*CAR, carboxylic acid reductase from *M. marinum*; OAA, oxaloacetate; PDH, pyruvate dehydrogenase complex; *Se*FAD, fatty aldehyde decarbonylase from *S. elongatus*; TE, acyl-ACP thioesterase; Yat1, mitochondrial carnitine acetyltransferase. (*B*) Specialized lipogenic metabolic pathways are physically separated and enclosed in different cellular compartments. AcCoA, acetyl-CoA; α-KG, α-ketoglutarate; AT, acyltransferase; CIT, citrate; DH, hydroxyacyl-ACP dehydratase; DHAP, dihydroxyacetone phosphate; ER, enoyl-ACP reductase; FFA, free fatty acids; G3P, glyceraldehyde 3-phosphate; ICT, isocitrate; KR, ketoacyl-ACP reductase; KS, ketoacyl-ACP synthase; MalCoA, malonyl-CoA; OAA, oxaloacetate; PPT, phosphopantetheinyl transferase.

3.2 mg/L (Fig. 2*D*). On the other hand, when *Ab*AtfA was targeted to the ER and peroxisome, FAEE production was increased to 136.5 and 110.9 mg/L (Fig. 2 *A* and *B*), respectively, roughly a 15- to 20-fold increase compared with cytosolic *atfA* overexpression. Similarly, we found that alkane production increased to 16.8 mg/L when the *Ab*ACR1-*Pm*ADO gene cluster was targeted to the ER (Fig. 2 *C* and *D*). The less-pronounced increase in alkane production is probably due to the low enzyme activity of *Pm*ADO, which relies on the iron cluster-mediated, free radical-catalyzed reduction process (20). Intracellular acyl-CoA trafficking was also attempted by overexpression of a peroxisomal/mitochondrial carnitine acyltransferase, perCat2 (21, 22), and increased FAEE and fatty alkane production to 142.5 and 16.6 mg/L (Fig. 2 *B* and *D*), respectively, similar to the results obtained with ER targeting.

Interestingly, targeting *Ab*AtfA and *Ab*ACR1-*Pm*ADO to different cellular compartments (cytosol, ER, and peroxisome) led to distinct FAEE and alkane product profiles. As time progresses, ER targeting of *Ab*AtfA gives rise to relatively stable FAEE production (Fig. 2*B*), with relatively longer chain-length distribution (*SI Appendix*, Fig. S2); on the other hand, both peroxisome targeting and perCat2 overexpression yield deteriorated FAEE production (Fig. 2*B*), with FAEEs shifting to a shorter chain-length range (*SI Appendix*, Fig. S2). Because the ER is spatially aligned with lipid-body formation, targeting of *Ab*AtfA to the ER may facilitate incorporation of FAEEs into lipid bodies and thus lead to relatively stable FAEE production. In contrast, the peroxisome contains multiple degradation and oxidation pathways that cleave long-chain acyl-CoAs into shorter-chain acyl-CoAs (15, 23), which could explain the presence of short-chain FAEEs in the engineered strain. The fatty alkane profile resembles the lipid fatty acid compositions in *Y. lipolytica*, except that peroxisome targeting or perCat2 overexpression leads to a relatively high portion of tridecane (Fig. 2*D*), possibly due to the presence of shorter-chain myristyl-CoA in the peroxisome due to β-oxidation activity. Cytosolic expression of *Mycobacterium marinum* carboxylic acid reductase *Mm*CAR (24) along with an ACP activation module, *Bsu*Sfp [phosphopantetheinyl transferase from *Bacillus subtilis* (19)], and *Pm*ADO resulted in an alkane production of 23.3 mg/L (Fig. 2*D*), suggesting that the endogenous fatty acid pool may be an alternative route to synthesizing alkane fuels.

Medium Chain-Length Fatty Acid Production in *Y. lipolytica*. We next investigated various strategies to modulate fatty acid chain length. Medium chain-length fatty acids (mclFAs) are of particular interest due to their higher carbon-conversion yield (*SI Appendix*, Fig. S3) and lower freezing/cloudy point (*SI Appendix*, Fig. S4) (1, 25). They are also precursors in the synthesis of various commodity chemicals. Fatty acyl-ACP/ acyl-CoA thioesterase (2) has been widely used in bacterial systems to modulate fatty acid chain length. We thus tested a panel of thioesterases with distinct chain-length specificity, but initial screening only resulted in a moderate increase in mclFAs (C12 up to 2.3% in *Cocos nucifera* *Cn*FatB2, and C14 up to 19.2% in *Escherichia coli* *Ec*YbgC) (*SI Appendix*, Fig. S5). We also attempted to target the bifunctional acyl-ACP/acyl-CoA

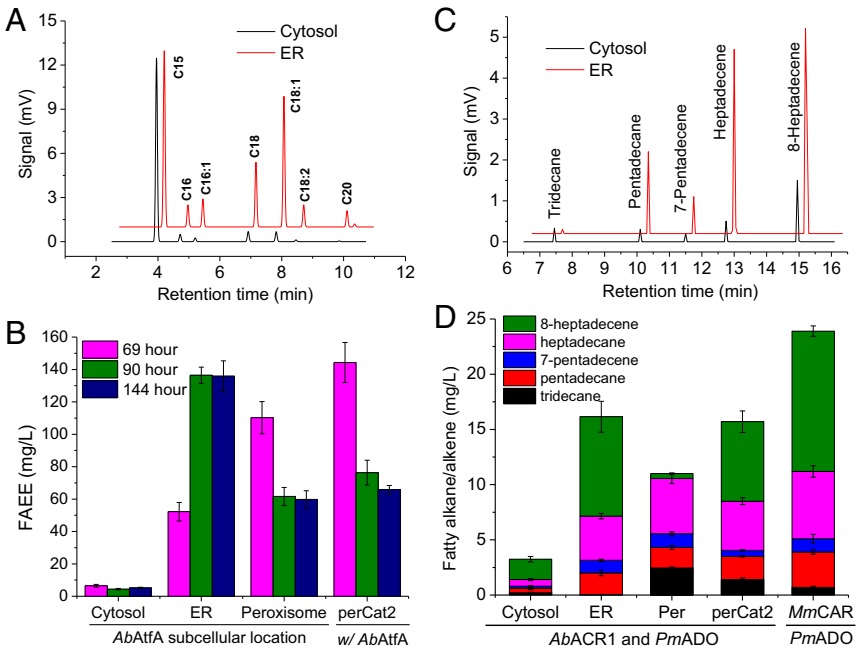

Fig. 2. Subcellular localization of acyl-CoA processing enzymes to produce FAEEs and fatty alkanes. (*A*) Gas chromatography profile of FAEEs obtained from *Y. lipolytica* when *Ab*AtfA is targeted to the ER (red) or cytosol (black). *n*-pentadecanoic acid (C15) ethyl ester was used as the internal standard. (*B*) FAEE production from *Y. lipolytica* with *Ab*AtfA expressed at different subcellular localizations. (*C*) GC profile of fatty alkanes obtained from *Y. lipolytica* with *Ab*ACR1 and *Pm*ADO targeted to either the cytosol (black) or ER (red). (*D*) Fatty alkane production from *Y. lipolytica* with *Ab*ACR1, *Pm*ADO, and *Mm*CAR expressed at different subcellular localizations. (*B* and *D*) Error bars represent SDs from three replicates.

thioesterase (*Ec*TesA′) to different cellular compartments; however, this approach also failed to further increase the fraction of mclFAs in the total fatty acid pool (*SI Appendix*, Fig. S6). This prompted us to investigate the catalytic mechanism and structure of the fatty acid synthase (FAS) complex in *Y. lipolytica*. Unlike the bacterial FAS complex that is composed of discrete, multiple subunits (normally five or six individual enzymes) (*SI Appendix*, Fig. S7), fungal FAS is encoded by two polypeptides, with each of them performing multiple functions (*SI Appendix*, Fig. S8). This structural difference increases the flexibility of bacterial FAS and allows exogenous thioesterases to easily access and hydrolyze the fatty acyl-ACP intermediates, offering the possibility to generate free fatty acids with tailored chain length in bacteria.

However, the fatty acid synthase complex (FAS1 and FAS2) in fungi forms a compact molecular cage that is dedicated to load and elongate the backbone of the acyl-ACPs (26). The MPT (malonyl/palmitoyl transacylase) tunnel-like domain of FAS1 (27) only allows the 16-carbon palmitoyl moiety to contact the transacylase domain (other shorter-chain acyl-ACPs are trapped in the tunnels), thus transferring the ACP group from palmitoyl-ACP to malonyl-CoA and leading to the formation of malonyl-ACPs and palmitoyl-CoAs (28) (palCoAs) (*SI Appendix*, Fig. S8). palCoA is further translocated to the ER and undergoes chain elongation and desaturation to initiate lipid biosynthesis. Any acyl-ACPs with a chain length of less than 16 will be trapped inside the FAS1 tunnel-like domain. This structural complexity restricts the exposure of shorter acyl-ACP intermediates to thioesterase hydrolysis and warrants the accurate and proper function of FAS in fungi. This partially explains the observation that only smaller thioesterases (EGTE 26 kDa, *Ec*TesA′ 20.7 kDa, *Ec*TesC 15.1 kDa, *Ec*YbgC 15.6 kDa, and *Ec*YciA 14.2 kDa) (*SI Appendix*, Fig. S5) give rise to better chain specificity, as smaller thioesterases may diffuse into the FAS cavity and hydrolyze acyl-ACPs to mclFAs. To bridge this gap, we swapped the MPT domain in FAS1 (*SI Appendix*, Fig. S9) and fused the truncated FAS1 with putative thioesterases that have been reported to cover a broad range of chain specificity. The resulting hybrid hFAS-TEs led to remarkably increased C12 and C14 portions of fatty acids (Fig. 3*A*). For example, expression of hFAS-*Ec*TesA′ increased C14 fatty acids (myristic acid) to 29.2%, almost a threefold increase relative to the cytosolic expression of TesA′ (*SI Appendix*, Fig. S5). Similarly, hFAS-UcBTE expression led to a sixfold increase of C12 fatty acids (increased to 7.5% from 1.2%) in the modified fungal FAS system (Fig. 3*A*). The strain with hybrid hFAS-*Ec*TesA′ overexpression yielded 1.3 g/L of free fatty acids in shake flasks, and this level was increased to 9.67 g/L upon scaling up to a 3-L bioreactor with pH control (*SI Appendix*, Fig. S10).

Recently, *Saccharomyces cerevisiae* has been extensively investigated to produce short-chain fatty acids and oleochemicals. Similar to *Y. lipolytica* FAS, researchers have developed alternative strategies to alter *S. cerevisiae* FAS chain specificity, including using orthogonal fatty acid synthases to produce fatty acids (29, 30) or oleochemicals (31, 32) with varied chain length. Incorporation of the amino acid degradation pathway with fatty acid synthase allowed the production of short branched-chain fatty acids (33) and branched-chain alkyl esters (34). Here we harnessed the modularity of the fungal fatty acid synthase and engineered a hybrid protein that retains the efficiency of the fungal acyl-ACP elongation machinery while accommodating acyl-ACPs with different chain lengths.

Fatty Alcohol Production in *Y. lipolytica*. Fatty alcohols, an important member of the oleochemcial family, have recently attracted particular interest due to their broad applications in the cosmetics, detergent, surfactant, and lubricant industries (25). We assessed various pathway engineering strategies to produce fatty alcohols in *Y. lipolytica* (*SI Appendix*, Fig. S11). ER targeting of *Ab*ACR1 and *E. coli* aldehyde reductase *Ec*AHR led to accumulation of 49.2 mg/L fatty alcohols (Fig. 3*B*). Coexpression of *Synechococcus elongatus* fatty acyl-ACP reductase *Se*FAR and *Ec*AHR resulted in less than 2.5 mg/L fatty alcohols, possibly indicating that there is an insufficient amount of acyl-ACPs

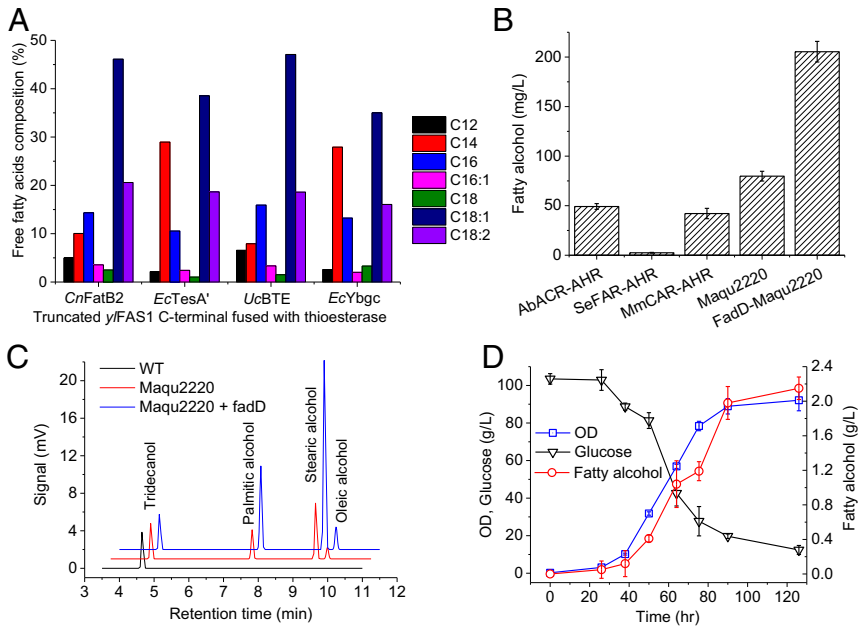

Fig. 3. Production of free fatty acids and fatty alcohols in *Y. lipolytica*. (*A*) Genetically altering the fatty acid chain-length specificity by engineering a hybrid fatty acid synthase in *Y. lipolytica*. (*B*) Screening of possible biosynthetic routes to produce fatty alcohols in *Y. lipolytica*. (*C*) Representative GC profile of a fatty alcohol-producing strain. Tridecanol (C13 alcohol) is the internal standard. Black, wild-type strain; red, *Y. lipolytica* with fatty acyl-CoA reductase Maqu2220 overexpression; blue, *Y. lipolytica* with Maqu2220 and *E. coli* fadD. (*D*) Scaled-up cultivation of a fatty alcohol-producing strain in a 3-L bench-top bioreactor. (*B* and *D*) Error bars represent SDs from three replicates.

that could be accessed by *Se*FAR in the cytoplasm. Cytosolic overexpression of the *M. marinum* carboxylic acid reductase [*Mm*CAR (24)] along with its activation partner BsuSfp and *Ec*AHR led to comparable fatty alcohol production at 42.1 mg/L. Interestingly, expression of the *Marinobacter aquaeolei* fatty acyl-CoA reductase Maqu2220 (35) along with an *E. coli* fatty acyl-CoA synthetase (*Ec*fadD) that activates free fatty acids to acyl-CoAs led to dramatic fatty alcohol titer improvement at 205.4 mg/L, a 2.6-fold increase compared with the single overexpression of Maqu2220 (Fig. 3*B*). These results suggested that there is a limited amount of cytosolic acyl-CoAs for downstream reduction; activating the endogenous fatty acids to acyl-CoAs by overexpression of fadD is sufficient to provide acyl-CoAs and push fatty alcohol production. The produced alcohols are primarily intracellular compounds and contain 60.6% stearic alcohol, 23.8 palmitic alcohol, and 15.6% oleic alcohol (Fig. 3*C* and *SI Appendix*, Fig. S12). Scale-up of the engineered Maqu2220-*Ec*fadD strain in a 3-L bioreactor further elevated fatty alcohol production to 2.15 g/L (Fig. 3*D*), albeit a prolonged cell-growth phase was observed, possibly due to alcohol toxicity. This result suggests that cytosolic activation of free fatty acids to acyl-CoAs is critical to fatty alcohol production in oleaginous yeast. Compared with the *S. cerevisiae* host (36), our engineered *Y. lipolytica* strain produced two times more fatty alcohols, demonstrating that *Y. lipolytica* is a superior host to produce long-chain alcohols and other oleochemicals.

Rewiring Acetyl-CoA Pathways to Improve Lipid Production in *Y. lipolytica*. The lipogenic potential of *Y. lipolytica* relies on the high acetyl-CoA flux, a distinct feature that makes this yeast produce large quantities of lipids. Instead of using acetyl-CoA synthase to provide cytosolic acetyl-CoAs (i.e., *S. cerevisiae*) (37), *Y. lipolytica* harnesses the ATP citrate lyase (ACL) to provide the acetyl-CoA building blocks for fatty acid synthesis. In *Y. lipolytica*, acetyl-CoA is solely produced by the ACL splitting of citrate when the TCA cycle is repressed under nitrogen-starvation conditions (9, 13). Because nitrogen is an essential nutrient for maintaining cell growth, the association between lipogenesis and nitrogen starvation forces lipid production at a stationary phase, resulting in prolonged cultivation time and relatively low productivity. Considering that turnaround and operational time is critical to improving productivity and cost efficiency in industrial settings, we hypothesize that engineering alternative cytosolic acetyl-CoA pathways may decouple acetyl-CoA flux from nitrogen starvation and initiate lipid production during cell growth, thus resulting in a shorter fermentation time.

To this end, we engineered five alternative cytosolic acetyl-CoA pathways in *Y. lipolytica* (Fig. 4*A*). These pathways include the pyruvate-acetate route (encoded by pyruvate decarboxylase *pdc*, aldehyde dehydrogenase *aldH*, and the native acetyl-CoA synthase *acs*), pyruvate-aldehyde route (encoded by pyruvate decarboxylase *pdc* and CoA-acetylating aldehyde dehydrogenase *aad*), pyruvate formate lyase (encoded by *pflA* and *pflaB*), acetyl-CoA shuttling pathway (carnitine acetyltransferase Cat2), and nonoxidative pentose-phosphate pathway [encoded by phosphoketolase (PK) and phosphotransacetylase *pta*]. When these pathways were expressed in one of our highly engineered AD strains [*Y. lipolytica* Po1g overexpressing acetyl-CoA carboxylase and diacylglycerol transferase (9)], we were able to improve both the lipid titer and oil content, with perCat2 conferring the most improvement and ACL the least improvement (Fig. 4*B*), indicating that alternative cytosolic acetyl-CoA pathways indeed play a critical role in lipid biosynthesis. We also overexpressed the synthetic pathways PK-Pta to couple NADPH regeneration with acetyl-CoA formation (Fig. 4*A*), which resulted in the highest yield of 0.225 g/g (gram lipids per gram glucose) in shake flasks (*SI Appendix*, Table S1). Normally due to C/N restriction, the parental strain only accumulates lipids following nitrogen depletion within a narrow time window between 30 and 70 h. In contrast, the engineered strains start accumulating lipids at the very initial stage during growth and continue this process throughout the entire fermentation (*SI Appendix*, Figs. S13 and S14). This is probably due to the fact that the strains with alternative pathways for the supply of cytosolic acetyl-CoA are less sensitive to C/N ratio regulation. To validate this hypothesis, we tested the engineered strains under different C/N

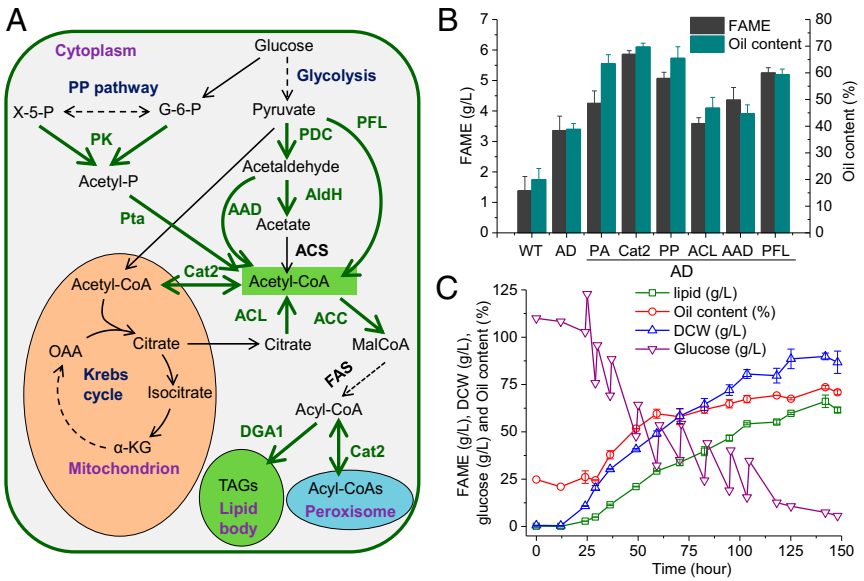

Fig. 4. Engineering pathways for the supply of cytosolic acetyl-CoA to improve lipid production in *Y. lipolytica*. (*A*) Alternative cytosolic acetyl-CoA pathways engineered in *Y. lipolytica*. AAD, CoA-acylating aldehyde dehydrogenase from *Pseudomonas putida*; ACS, acetyl-CoA synthase; AldH, aldehyde dehydrogenase; G-6-P, glucose 6-phosphate; PDC, pyruvate decarboxylase; PFL, pyruvate formate lyase; X-5-P, xylulose 5-phosphate. (*B*) Expression of alternative cytosolic acetyl-CoA pathways improves both lipid titer and oil content. AAD, PDC-AAD; AD, ACC-DGA; FAME, fatty acid methyl esters; PA, PDC-AldH; PP, PK-Pta. (*C*) Fed-batch cultivation of *Y. lipolytica* AD expressing perCat2 under controlled conditions. Sixty milliliters of 400 g/L glucose was pulsed into the bioreactor every 8 or 10 h. DCW, dry cell weight. (*B* and *C*) Error bars represent SDs from three replicates.

ratios. The lipid titer obtained from the engineered strains declined much more slowly compared with the parent AD strain (*SI Appendix*, Figs. S15 and S16) as we lowered the C/N ratio, suggesting that alternative acetyl-CoA pathways indeed were functional in alleviating ACL regulation and pushing more acetyl-CoA to lipid synthesis regardless of the high nitrogen concentration. With this, we tested the best performer, the strain with Cat2 overexpression, in a 3-L bioreactor under controlled glucose feeding and constant pH conditions (Fig. 4*C*). The engineered strain achieved a dry cell weight of 91.6 g/L and lipid titer of 66.4 g/L with a process yield at 0.229 g/g and productivity at 0.565 $g{\cdot}L^{-1}{\cdot}h^{-1}$ (Fig. 4*C*). Thus, engineering cytosolic acetyl-CoA pathways has effectively synchronized lipid production with cell growth and led to an ∼3.1-fold increase in lipid productivity over the parental strain (9).

Conclusions

Global energy demand and environmental concerns have called worldwide attention to the production of carbon-neutral fuels and bioproducts directly from renewable resources. Among the investigated chassis organisms, oleaginous yeasts have been shown to accumulate very high levels of neutral lipids. The work reported here demonstrates that *Y. lipolytica* represents a promising biorefinery platform for sustainable production of drop-in transportation fuels and oleochemicals. Understanding the mechanistic details of the lipogenic phenotype, particularly the cellular compartmentalization of distinct metabolic pathways, fatty acid synthase structure, activating free fatty acids to acyl-CoAs, and decoupling nitrogen starvation from lipogenesis, allowed us to efficiently produce fatty acid ethyl esters, fatty alkanes, medium chain-length fatty acids, fatty alcohols, and triacylglycerides. Our engineering strategies have led to *Y. lipolytica* strains producing 142.5 mg/L FAEEs, 23.3 mg/L fatty alkanes, 2.15 g/L fatty alcohols, 9.67 g/L free fatty acids (with 7.5% C12 and 29.2% C14), and 66.4 g/L TAGs. The products obtained in this study may serve as biorefinery platform chemicals for sustainable production of diesel fuels and oleochemicals via simple chemical or biotransformation. Future efforts focusing on further strain improvement, bioprocess optimization, and genome evolution should aid the development of more robust processes to achieve high titer, yield, and productivity. We envision that this report constitutes a foundational work in developing an oleaginous yeast platform to upgrade low-value carbons to high-value fuels and oleochemicals in the foreseeable future.

Methods

Yeast Strains, Growth, and Culture Conditions. The *Y. lipolytica* wild-type strain W29 was purchased from ATCC (ATCC20460). The auxotrophic Po1g (Leu−) was obtained from Yeastern Biotech. All strains and plasmids used in this study are listed in *SI Appendix*, Table S2.

Y. lipolytica shake-flask culture is listed in *SI Appendix, Methods*. Fermenter experiments were carried out in a 3-L BioFlo stirred-tank bioreactor equipped with New Brunswick BioCommand control systems (Eppendorf). The media contained 3.4 g/L yeast nitrogen base (without amino acids and ammonium sulfate), 2.2 g/L ammonium sulfate, and 100 g/L glucose. Specifically, 120-mL shake-flask culture (harvested at 48 h) was used as seed to inoculate 1 L fermentation media (with C/N 100). Dissolved oxygen was controlled at 20% through cascading with agitation. Sterile air was sparged into the bioreactor at a fixed volume ratio (2 vol/vol/min) and the pH was controlled at 5.5 through feeding 6 N NaOH. Temperature was controlled at 28 °C, and 400 g/L glucose was fed into the bioreactor when the bioreactor was run in a fed-batch mode. Fermenter experiments were performed in duplicate.

Self-Replicative Plasmid Construction and Modular Pathway Assembly. To facilitate gene cloning and assembly, a self-replicative and assembly-friendly *Yarrowia* vector was constructed to accommodate centromere and replication origin (38, 39) as well as the ePathBrick gene assembly feature (40). Specifically, a gene fragment containing centromere 1.1 (CEN 1.1), TEF promoter, exon, incomplete intron, multiple cloning sites, XPR2 terminator, and replication origin ORI1001 was synthesized by Invitrogen (the first gene fragment in *SI Appendix*, Table S4). Then, this synthetic fragment was digested with ClaI and XhoI and ligated to ClaI- and SalI-digested pMT015 (9) (note: XhoI and SalI are compatible ends) to give the vector pYLXP′. Then, the additional SpeI on the pYLXP′ plasmid backbone was mutated with the primers Spe_SF and Spe_SR using site-directed mutagenesis PCR to give the vector pYLXP. With pYLXP as a platform vector, all of the structural genes were cloned into this vector backbone.

Oleochemical biosynthetic pathway gene fragments were synthesized by Life Technologies; detailed sequences are listed in *SI Appendix*, Table S4. Selected acetyl-CoA pathway gene targets were amplified either from *E. coli* K-12, *S. cerevisiae* S288c, or *Y. lipolytica* W29 genomic DNA with Q5 Hot Start DNA polymerase (New England Biolabs). All of the PCR products and synthetic

gene fragments were designed on either end to contain an ~35-bp region that is homologous to the plasmid backbone to facilitate Gibson assembly. Purified PCR gene products or synthetic gene fragments were assembled with the SnaB1- and KpnI-digested pYLXP plasmid backbone using Gibson assembly. Recombinant plasmids from positive transformants were miniprepped and verified by double digestion. Plasmids with the correct digestion pattern were further sequenced to confirm the insertion of the correct gene fragment. All sequencing services were provided by Quintara Biosciences. To assemble multiple genes on the same vector, an AvrII- and SalI-digested donor vector containing the donor gene was purified and ligated to the NheI- and SalI-digested recipient vector using T4 DNA ligase (New England Biolabs) to give the construct coexpressing two genes, with each of the genes in the promoter–gene cassette–terminator configuration. Occasionally, BglII, NotI, or ClaI sites were used to substitute for SalI in cases where SalI would cut the structural gene. This assembly procedure can be iteratively used to efficiently create plasmid constructs expressing multiple genes (up to six or more) (40). Genetic manipulation for thioesterase cloning, addition of ER retention signal and peroxisome targeting signal, and creation of hybrid fatty acid synthase–thioesterase fusion protein are described in *SI Appendix, Methods*.

All constructed plasmids in this study are listed in *SI Appendix*, Table S2. Constructed vectors were transformed into *Y. lipolytica* Po1g Δleu or Po1g ΔleuΔura strains following the protocol reported by Chen et al. (41) and grown on CSM-leu or CSM-leu-ura plates for transformant screening.

Lipid, FAEE, Fatty Alkane, mclFA, and Fatty Alcohol Extraction and Analysis. To quantify lipid titer and fatty acid composition, 4 OD units of harvested cell pellet (without fermentation broth) was directly saponified with 0.5 M sodium methoxide and followed by vortexing vigorously at 1,200 rpm for 2 h. Then, 40 μL 98% sulfuric acid was added to neutralize the sample. Caution must be taken when adding the sulfuric acid; expect a violent exothermic reaction. Hexane (400 μL) was added to extract the fatty acid methyl esters. The hexane extractions were vortexed at 1,200 rpm for 10 min and centrifuged at 11,000 × *g* for 2 min. Hexane extracts (250 μL) were withdrawn and injected onto gas chromatography–flame ionization detector (GC-FID) to quantify fatty acids and lipid titer. To account for extraction and transesterification efficiency, 100 μL of 2 g/L of tridecanoate methyl ester and 2 g/L of glyceryl triheptadecanoate were supplemented as internal standards at the very beginning. Detailed procedures for quantifying FAEE, fatty alkane, and glucose levels and dry cell weight can be found in *SI Appendix, Methods*.

For fatty alcohol extraction, 3 mL cell culture was supplemented with 10 μL of 10 mg/mL tridecanol, acidified with 100 μL HCl, and directly extracted with 2 mL chloroform/methanol (vol/vol, 2:1) in a 7-mL sealed glass vial with occasional shaking in a 60 °C oven overnight. After centrifugation at 4,000 × *g* for 5 min to separate the organic and water layers, 600 μL of the chloroform phase was transferred to an Eppendorf tube and dried under air and reconstituted with 350 μL hexane. The hexane phase was directly injected onto GC-FID for fatty alcohol analysis. mclFA extraction followed the same procedure as for fatty alcohols, except that the chloroform extracts (dried extracts) were mixed with 600 μL 5% sulfuric acid/methanol in a 60 °C oven overnight. After transesterification, the samples were extracted with 600 μL hexane and the hexane phase was injected onto GC-FID with pentadecanoate methyl ester as an internal standard to analyze the fatty acid methyl esters. FAEE, fatty alkane, mclFA, and fatty alcohol samples were also injected onto a Varian Saturn 2000 GC-MS to confirm the structure. The GC-FID temperature profile is listed in *SI Appendix, Methods*.

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