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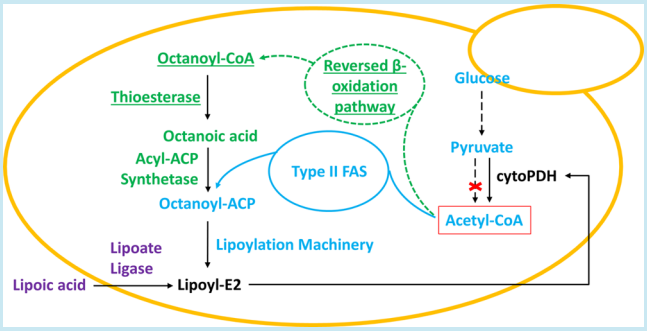
Functional Reconstitution of a Pyruvate Dehydrogenase in the Cytosol of *Saccharomyces cerevisiae* through Lipoylation Machinery Engineering

Jiazhang Lian[†] and Huimin Zhao*[,†,‡]

[†]Department of Chemical and Biomolecular Engineering, Institute for Genomic Biology, and [‡]Departments of Chemistry, Biochemistry, and Bioengineering, University of Illinois at Urbana−Champaign, Urbana, Illinois 61801, United States

Supporting Information

ABSTRACT: Acetyl-CoA is a key precursor for the biosynthesis of a wide range of fuels, chemicals, and value-added compounds, whose biosynthesis in *Saccharomyces cerevisiae* involves acetyl−CoA synthetase (ACS) and is energy intensive. Previous studies have demonstrated that functional expression of a pyruvate dehydrogenase (PDH) could fully replace the endogenous ACS-dependent pathway for cytosolic acetyl-CoA biosynthesis in an ATP-independent manner. However, the requirement for lipoic acid (LA) supplementation hinders its wide industrial applications. In the present study, we focus on the engineering of a *de novo* synthetic lipoylation machinery for reconstitution of a functional PDH in the cytosol of yeast. First, a LA auxotrophic yeast strain was constructed through the expression of the *Escherichia coli* PDH structural genes and a lipoate−protein ligase gene in an ACS deficient (*acs1*Δ *acs2*Δ) strain, based on which an *in vivo* acetyl-CoA reporter was developed for following studies. Then the *de novo* lipoylation pathway was reconstituted in the cytosol of yeast by coexpressing the yeast mitochondrial lipoylation machinery genes and the *E. coli* type II fatty acid synthase (FAS) genes. Alternatively, an unnatural *de novo* synthetic lipoylation pathway was constructed by combining the reversed β-oxidation pathway with an acyl-ACP synthetase gene. To the best of our knowledge, reconstitution of natural and unnatural *de novo* synthetic lipoylation pathways for functional expression of a PDH in the cytosol of yeast has never been reported. Our study has laid a solid foundation for the construction and further optimization of acetyl-CoA overproducing yeast strains.



KEYWORDS: *pyruvate dehydrogenase, protein lipoylation, acetyl-CoA, synthetic biology, yeast*

Besides its significant role as a building block in cellular metabolism, acetyl-CoA is the key precursor for biological synthesis of a variety of fuel and chemical molecules, such as *n*-butanol, fatty acid ethyl esters (FAEEs), alkanes, polyhydroxybutyrate (PHB), and isoprenoid-derived drugs.[1−8] However, *Saccharomyces cerevisiae* is not optimized to synthesize acetyl-CoA, in terms of the titer and yield of acetyl-CoA derived molecules, if compared with those in *Escherichia coli*. One explanation is the difference in acetyl-CoA metabolism. In *E. coli*, acetyl-CoA is steadily synthesized from pyruvate by pyruvate dehydrogenase (PDH)[9] or pyruvate:formate lyase (PFL).[10] In eukaryotes, the metabolism of acetyl-CoA is separated into several compartments, including the mitochondria, peroxisomes, nucleus, and cytosol.[11] Acetyl-CoA is mainly generated in the mitochondria by PDH; however, *S. cerevisiae* lacks the machinery to export the mitochondrial acetyl-CoA to the cytosol where biosynthesis of the desired products generally occurs.[11] In the cytosol of yeast, acetyl-CoA is generated via the PDH-bypass pathway, from pyruvate to acetaldehyde and then to acetate, which is activated to acetyl-CoA by the acetyl-CoA synthetase (ACS) at the cost of two ATP equivalents. Because of the feedback inhibition of ACS and high energy input requirement, the activation of acetate is a rate-limiting step.[7] In addition, acetaldehyde is the branch point to control the flux to ethanol and acetyl-CoA, and most of the metabolic fluxes are going to ethanol during glucose fermentation due to the Crabtree effect and the lower affinity of aldehyde dehydrogenases (ALDHs) toward acetaldehyde than that of alcohol dehydrogenases (ADHs). Therefore, the ACS-dependent acetyl-CoA biosynthesis in the cytosol of *S. cerevisiae* suffers from low metabolic fluxes and low efficiencies.

PDH catalyzes the oxidative degradation of pyruvate to generate acetyl-CoA in an ATP-independent manner, which is generally adopted and one of the most efficient routes for acetyl-CoA generation in nature. PDH is a huge multisubunits protein complex, with thiamine pyrophosphate (TPP), FAD, NAD^+, and lipoic acid (LA) used as cofactors. Since TPP, FAD, and NAD^+ are readily available in the cytosol of yeast, the supply of LA as well as its attachment to PDH (lipoylation of E2 subunit) should be engineered together with the expression

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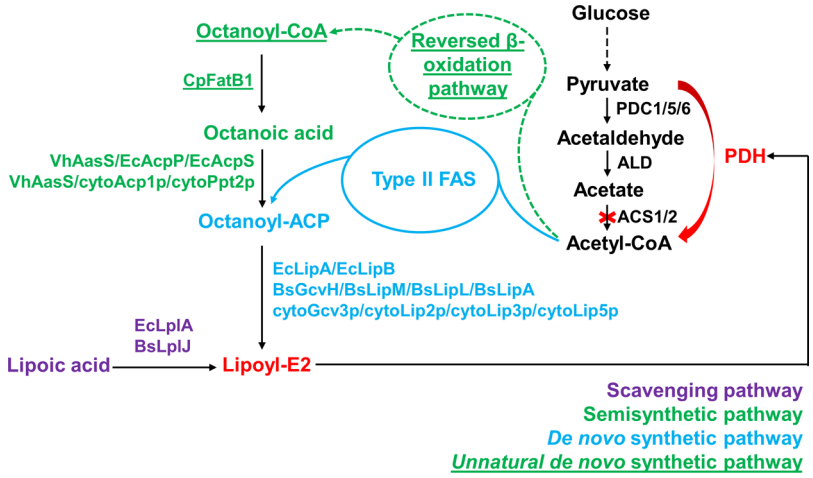

Figure 1. Overview of acetyl-CoA biosynthesis and lipoylation machinery engineering for a functional PDH in the cytosol of *S. cerevisiae*. Functional PDH (shown in red) was designed and engineered with different lipoylation machineries, including the scavenging pathway (purple), the semisynthetic pathway (green), and the *de novo* synthetic pathway (blue). An unnatural *de novo* synthetic lipoylation pathway (green and underlined) was also designed by integrating the semisynthetic pathway with the reversed *β*-oxidation pathway. The endogenous ACS-dependent acetyl-CoA biosynthesis (black) was abolished by the deletion of *ACS1* and *ACS2* and the activity of PDH was evaluated by its ability to rescue the growth on glucose synthetic medium.

of PDH structural genes. In nature, two independent routes are probably synergistically used for protein lipoylation, the scavenging pathway and the *de novo* synthetic pathway. As for the scavenging pathway, exogenous LA is assimilated by the host and attached to its protein targets by a lipoiate−protein ligase (Lpl). When exogenous LA is not available, it has to be synthesized *de novo* from octanoyl-ACP (Figure 1), an intermediate of the Type II fatty acid biosynthesis. In *E. coli* (Supplementary Figure 1A), where the lipoylation pathway is the most well-characterized, the *de novo* synthetic pathway proceeds by two consecutive reactions: in the first step the octanoyltransferase (LipB) transfers the octanoyl moiety from the endogenously produced octanoyl-ACP to the target apoproteins; and in the second step the octanoyl moiety is converted to LA by the lipoyl synthase (LipA) to generate lipoylated proteins.[12] In *Bacillus subtilis* (Supplementary Figure 1B), a Gram-positive bacterium, it is interesting to find that a lipoylated protein (GcvH) is required for the lipoylation of PDH. Later it is found that LipM is an octanoyltransferase that specifically transfers the octanoyl moiety from octanoyl-ACP to GcvH, whereas LipL is an amido-transferase that transfers the octanoyl moiety from GcvH to PDH.[13,14] In *S. cerevisiae*, the lipoylation pathway is less understood, which is also found to be Gcv3p-dependent,[15−17] and a mechanism similar to that of *B. subtilis* has been proposed (Supplementary Figure 1B).

Considering the limitations of the endogenous ACS-dependent pathway for acetyl-CoA generation, we sought to characterize alternative biosynthetic pathways with higher efficiency and lower energy input requirement via synthetic biology with a focus on the PDH from *E. coli*. Recently, functional expression of the PDH complex in the cytosol of yeast was achieved by coexpressing the PDH structural genes (*pdhA*, *pdhB*, *aceF*, and *lpd*) and the protein-lipoate ligase genes (*lplA* and *lplA2*) from *Enterococcus faecalis*. When LA was supplemented to the growth medium, a functional PDH complex could fully replace the endogenous ACS-dependent pathway for cytosolic acetyl-CoA synthesis.[18] Although a growth rate similar to the wild-type yeast strain was achieved, the requirement of LA supplementation and its high cost hinder further application of such a system in industry. Therefore, in this study, we focus more on the *de novo* biosynthetic lipoylation pathway, besides the scavenging pathway, to reconstitute a functional PDH complex in the cytosol of yeast. First of all, a LA auxotrophic yeast strain was constructed by reconstitution of the scavenging pathway, based on which we developed an *in vivo* acetyl-CoA reporter. As an intermediate step to *de novo* lipoylation, a semisynthetic lipoylation pathway was designed to provide the lipoylation substrate octanoyl-ACP by the acyl-ACP synthetase (AasS) and exogenously supplemented octanoic acid. Coexpression of Vh*AasS*-cyto*ACP1*-cyto*PPT2* together with the yeast mitochondrial lipoylation machinery in the cytosol resulted in a functional PDH and rescued the growth of the Acs$^-$ strain when octanoic acid was supplemented. On the basis of these results, an unnatural *de novo* biosynthetic lipoylation pathway was designed and constructed by integrating the reversed *β*-oxidation pathway to provide octanoic acid for the semisynthetic lipoylation pathway. Finally, the *de novo* lipoylation pathway was reconstituted in the cytosol by coexpressing the yeast mitochondrial lipoylation machinery genes and the type II FAS genes from *E. coli*, which resulted in a functional PDH and enabled the growth of the Acs$^-$ strain with glucose as the sole carbon source. To the best of our knowledge, it is the first report on the reconstitution of a semisynthetic, a *de novo* synthetic, and an unnatural *de novo* synthetic lipoylation pathways for functional expression of a PDH in the cytosol of yeast.

RESULTS AND DISCUSSION

PDH with a Scavenging Lipoylation Pathway. Although several protocols for the detection of CoAs in biological systems have been developed, it is still rather tedious and labor intensive to perform the extractions and assays.[19,20] Thus, an *in vivo* acetyl-CoA assay system was developed by disrupting the endogenous ACS-dependent acetyl-CoA biosynthesis. In *S. cerevisiae*, Acs1p and Acs2p are responsible for the activation of acetate (ACS activities). While *ACS2* is found to be constitutively expressed under all conditions, the expression of *ACS1* is repressed by the presence of glucose.[1,18] In other

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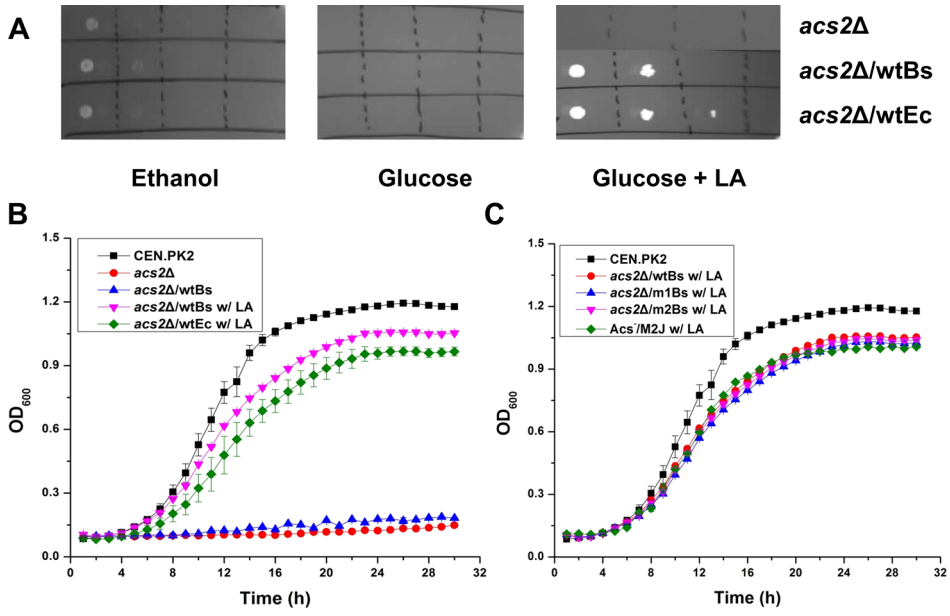

Figure 2. Complementation of the cell growth of *acs2*Δ yeast strain by pyruvate dehydrogenase (PDH). The cell growth was determined by either spotting assays (A) on synthetic medium agar plates containing ethanol, glucose, and glucose with LA, respectively, or growth curves (B and C) in the corresponding liquid media with or without 100 μg/L LA using the BioScreen C. Error bars represented SD of biological quadruplicates.

words, the disruption of *ACS2* would result in a yeast strain that could not grow on synthetic medium with glucose as the sole carbon source (Figure 2A). Since *ACS1* was expressed well under derepressed conditions, the disruption of *ACS2* did not have a significant impact on the growth on ethanol containing medium (Figure 2A). Therefore, the acetyl-CoA biosynthetic pathways could be characterized by their ability to complement the growth of the *acs2*Δ *S. cerevisiae* strain on glucose synthetic medium. After restoring the growth with alternative acetyl-CoA biosynthetic pathways, *ACS1* should be further deleted to eliminate any possibility of host adaptation that might lead to the constitutive expression of *ACS1* when glucose was still present.

As shown in Figure 2A, coexpression of *E. coli* PDH structural genes (Ec*LpdA*-Ec*AceE*-Ec*AceF*) and a lipoate-protein ligase gene (*LplA* from *E. coli* or *LplJ* from *B. subtilis*) could complement the growth of the *acs2*Δ *S. cerevisiae* strain on glucose synthetic medium, when LA was supplemented. Interestingly, the lipoate-protein ligase from *B. subtilis* (BsLplJ) performed better than that from *E. coli* (EcLplA) when coexpressed with the *E. coli* PDH structural genes (Figure 2B). Further disruption of *ACS1* resulted in no significant difference in the strain phenotypes (Figure 2C). Compared with the wild-type (Acs^+) strain, the growth rate and biomass yield were quite similar, indicating that PDH could fully replace the endogenous ACS-dependent pathway for cytosolic acetyl-CoA biosynthesis. Without LA supplementation, no significant cell growth could be observed (Figure 2A,B). In other words, the PDH containing Acs^- strain was LA auxotrophic.

PDH Mutants Insensitive to NADH Inhibition. The *E. coli* PDH was reported to subject to feedback inhibition by NADH, which might lead to decreased activity under anaerobic conditions (high ratio of NADH/NAD^+). Inverse engineering revealed that a mutation in the Ec*LpdA* coding sequence (E354 K) resulted in an NADH feedback inhibition resistant PDH mutant.[21] A subsequent study on the saturation mutagenesis of the E354 site found more PDH mutants that were insensitive to NADH inhibition.[22] In other words, these PDH mutants were functional under both aerobic and anaerobic conditions. Considering the reducing environment in the cytosol of yeast and the preference of anaerobic conditions for industrial applications, two feedback inhibition insensitive PDH mutants containing the mutation of Ec$LpdA^{E354K}$ (PDHm1) or Ec$LpdA^{E354G}$ (PDHm2) were constructed. As shown in Figure 2C, no significant difference in the growth rate of the *acs2*Δ strains containing either the wild-type or the mutated PDHs was observed, indicating that PDH activity was not inhibited under aerobic conditions. To test the advantage of using the NADH insensitive mutants, the PDH containing *acs2*Δ strains were cultured under anaerobic conditions. As shown in Supplementary Figure S2A,B, there was still no significant difference in the fermentation performance (glucose consumption and ethanol production) between these strains (20 g/L glucose), indicating that the NADH/NAD^+ ratio was not high enough in the yeast cytosol to inhibit the activity of PDH or the activity of PDH was still high enough to provide sufficient amount of acetyl-CoA even under inhibition conditions. Finally, fermentation with high concentration of glucose (100 g/L) was carried out under anaerobic conditions, which resulted in slightly improved fermentation performance with the NADH insensitive mutants (Supplementary Figure S2C,D). The results indicated that the reoxidation of NADH via ethanol and glycerol formation was able to maintain the cytosolic NADH/NAD^+ ratio at a low level that would not inhibit the activity of PDH, even under anaerobic conditions. Nevertheless, since no significant difference between PDHwt, PDHm1, and PDHm2 under aerobic conditions was observed and the PDH mutants might be more active under anaerobic conditions, especially in the engineered strains with impaired glycerol and ethanol fermentation, PDHm2 was chosen for all following studies to reconstitute the *de novo* synthetic lipoylation machinery.

Development of the Lipoic Acid Auxotrophic Strain as an *in Vivo* Acetyl-CoA Assay System. Although the *acs2*Δ *S. cerevisiae* strain has been used for *in vivo* acetyl-CoA assays, *ACS1* must be further deleted to confirm the function of

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the heterologous acetyl-CoA biosynthetic pathways. If the heterologous pathways were not very efficient in synthesizing acetyl-CoA, further disruption of *ACS1* in a poorly growing strain could be challenging. Therefore, we switched to the use of Acs$^-$/M2J, a LA auxotrophic strain, as the *in vivo* acetyl-CoA assay system. As shown in Figure 3, no significant cell growth was observed without LA supplementation, and the growth was increased gradually with higher LA concentrations. When the supplemented LA concentration was higher than 8 μg/L, a near wild-type growth rate could be achieved (Figure 3). Another advantage of the new acetyl-CoA assay system was the fast growth rate, compared with the *acs2*Δ *S. cerevisiae* strain, which had to be cultured on ethanol containing medium at an extremely slow growth rate. In addition, much higher transformation efficiency could be achieved (data not shown), making additional genetic modifications easier. Except for the final step of acetyl-CoA assays, which must be carried out in LA free medium, all previous steps including transformation and seed culture could be carried out in LA containing medium.

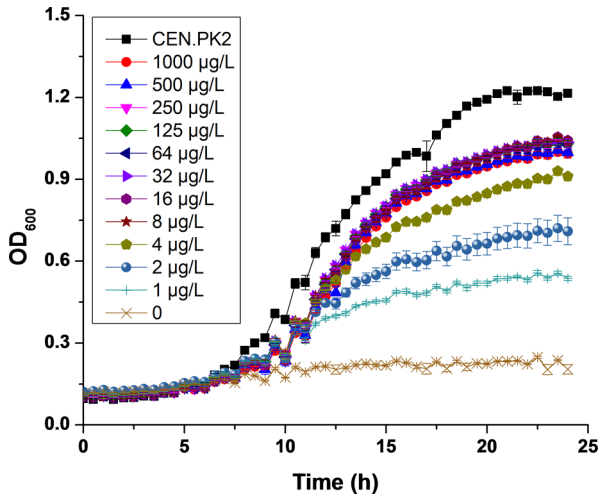

Figure 3. Growth dependence of Acs$^-$/M2J on lipoic acid concentration. Acs$^-$/M2J strain was pregrown in SCD-TRP-URA (SCD-WU) medium supplemented with 100 μg/L LA until saturation, washed in ddH_2O for three times, and then inoculated into fresh SCD medium supplemented with LA at the indicated concentrations with an initial OD of 0.1. The growth of CEN.PK2 was measured under the same conditions and included for comparison. Error bars represented SD of biological quadruplicates.

PDH with a Semisynthetic Lipoylation Pathway. Although a functional PDH was reconstituted in the cytosol of yeast, its industrial application would still be hindered by the high cost of LA. Therefore, we sought to reconstitute the *de novo* synthetic lipoylation pathway. Octanoyl-ACP, an intermediate of fatty acid biosynthesis, is the natural substrate of lipoylation. Unfortunately, the lipoylation enzymes have no access to octanoyl-ACP, which is constrained in the catalytic core of the type I FAS in the cytosol of yeast. In other words, a type II FAS should be functionally reconstituted in the cytosol for the *de novo* lipoylation machinery. To achieve this challenging goal step by step, a semisynthetic lipoylation pathway was proposed and designed by taking advantage of the *Vibrio harveyi* acyl-ACP synthetase (VhAasS), a soluble enzyme that ligates free fatty acids to ACP to form acyl-ACPs.[23,24] Interestingly, while most of the acyl-ACP synthetases are membrane proteins, VhAasS is a soluble cytosolic protein.[24] As there is no free ACP in the cytosol of yeast, ACP and its activator must be included together with VhAasS. The first strategy was to relocate the mitochondrial proteins (encoded by *ACP1* and *PPT2*, respectively) to the cytosol by removing the mitochondrial targeting sequences (cyto*ACP1* and cyto*PPT2*). Another strategy was to use the *E. coli* counterparts (encoded by Ec*AcpP* and Ec*AcpS*, respectively), which possessed no mitochondrial targeting sequences. In addition, the lipoylation pathways from *E. coli*, *B. subtilis*, and *S. cerevisiae* mitochondria were cloned and assembled, and the resultant DNA constructs were named as EcLA (Ec*LipA*-Ec*LipB*), BsLA (Bs*GcvH*-Bs*LipL*-Bs*LipM*-Bs*LipA*), and ScLA (cyto*GCV3*-cyto*LIP2*-cyto*LIP3*-cyto*LIP5*), respectively. As for ScLA, the mitochondrial targeting sequences were removed to relocate the full pathway enzymes to the cytosol of *S. cerevisiae*.

As shown in Figure 4A, no significant cell growth was observed for all strains when octanoic acid was missing, indicating that the activity of VhAasS was dependent on the presence of octanoate. If 10 mg/L octanoic acid was

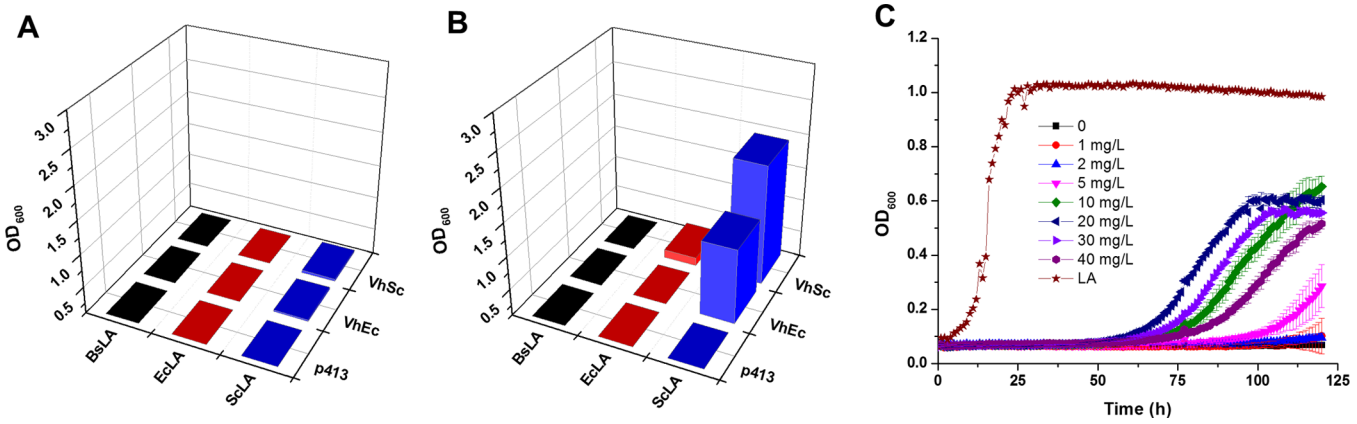

Figure 4. Reconstitution of a functional PDH by the semisynthetic lipoylation pathway. Recombinant yeast strains were precultured on SCD-HWLU medium supplemented with 100 μg/L LA, washed three times in ddH_2O, and inoculated into 5 mL of fresh SCD-HWLU medium in 14 mL round-bottom culture tubes without (A) or with (B) the supplementation of 10 mg/L octanoic acid. Cell densities were measured 5−7 days after inoculation. The results represented the average of biological duplicates, whose variation was less than 10%. (C) The concentration of octanoic acid was optimized for the growth of SemiLip (Acs$^-$/M2J+ScLA+VhSc) strain in SCD-HWLU medium. The growth of the SemiLip strain in SCD-HWLU medium supplemented with 100 μg/L LA was included as a positive control. Error bars represented the SD of biological quadruplicates.

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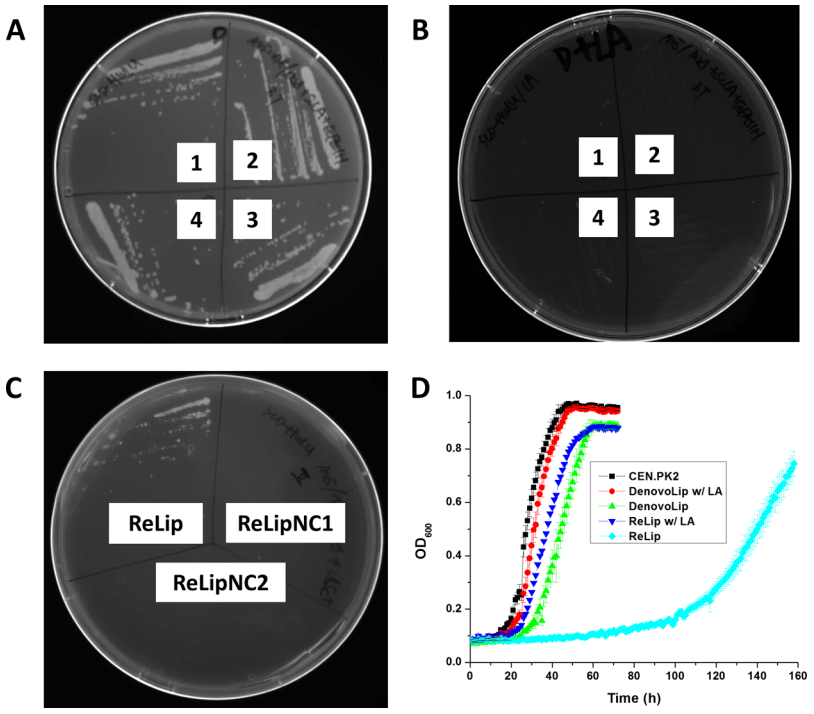

Figure 5. Reconstitution of a functional PDH by the natural and unnatural *de novo* synthetic lipoylation pathway. Plasmid stability assays of DenovoLip strain by serial transfers in SCD medium without (A) or with (B) the supplementation of LA. Four colonies were randomly picked up to evaluate the capability of maintaining the plasmids without auxotrophic selections. (C) Redesign of the *de novo* lipoylation pathway by combining the semisynthetic pathway with the reversed β-oxidation pathway (ReLip). The removal of either pathway (ReLipNC1 or ReLipNC2) abolished the ability to grow on glucose synthetic medium, indicating a lack of functional PDH. (D) Recombinant yeast strains were precultured on SCD-HWLU medium supplemented with 100 μg/L LA, collected by centrifugation, washed three times in ddH_2O, and then inoculated into fresh SCD-HWLU medium with or without the supplementation of 100 μg/L LA. The growth of the wild-type yeast strain containing the same plasmids was also measured and included for comparison. Error bars represented SD of biological quadruplicates.

supplemented to the growth medium, cell growth was observed in the Acs$^-$/M2J strain expressing ScLA and VhEc (Vh*AasS*-Ec*AcpP*-Ec*AcpS*) or VhSc (Vh*AasS*-cyto*ACP1*-cyto*PPT2*); little growth was observed for EcLA; and no significant growth was observed for BsLA (Figure 4B). The results indicated that BsLA was not active, EcLA showed little activity, while ScLA was functional in the cytosol of yeast and could accept ACP from both *E. coli* and *S. cerevisiae* mitochondria. The cell growth of Acs$^-$/M2J strains with semisynthetic lipoylation pathways was further confirmed in octanoic acid containing liquid medium, which was also compared with the strain containing a scavenging pathway (Figure 4C). Octanoic acid concentration of 10−30 mg/L was found to be optimal for cell growth: lower concentration led to low lipoylation efficiency and limited acetyl-CoA biosynthesis, while higher concentration resulted in cytotoxicity (Figure 4C). Since ScLA was the most active, it was used for the subsequent studies to reconstitute a *de novo* synthetic lipoylation pathway.

PDH with a *de novo* Synthetic Lipoylation Pathway. A final goal of this study is to design a *de novo* synthetic lipoylation pathway, that is, reconstitution of a functional PDH in synthetic medium without the supplementation of octanoic acid or LA. The type II FAS (Cem1p-Oar1p-Htd2p-Etr1p-Acp1p-Ppt2p-Mct1p) from the mitochondria of *S. cerevisiae* (Supplementary Figure S3), which was relocated to the cytosol by removing the mitochondrial targeting sequences (cyto-FAS1), was tested initially for the *de novo* synthetic lipoylation pathway. Unfortunately, no significant cell growth was observed with the coexpression of M2J and ScLA in the Acs$^-$ strain when cultured in synthetic medium with glucose as the sole carbon source (Supplementary Results and Discussion). Therefore, we next investigated the type II FAS from *E. coli*,[25] the most well-characterized FAS system, for *de novo* lipoylation in the cytosol of yeast.

When the type II FAS from yeast mitochondria was replaced by the *E. coli* system (EcAcpP-EcAcpS-EcFabD-EcFabH-EcFabF-EcFabG-EcFabA-EcFabI, Supplementary Figure S3), the Acs$^-$ strain grew readily in LA-free medium (Figure 5A,D), indicating the full reconstitution of the lipoylation machinery and the functional PDH in the cytosol of yeast. Although the growth rate is still lower than the wild-type yeast strain and that with the scavenging lipoylation pathway, it is the first report on the reconstitution of a *de novo* synthetic lipoylation machinery which paves the way for further optimization. To further confirm that the growth was due to the introduction of the *de novo* lipoylation machinery, plasmid stability assays were performed by serial transfers in SCD complete medium with or without the supplementation of LA. In LA free medium, the biosynthesis of acetyl-CoA itself functions as the selection pressure to maintain all the three plasmids, containing PDH genes, lipoylation genes, and type II FAS genes, respectively. On the contrary, when LA is supplemented, there was no selection pressure for the type II FAS plasmid (EcFAS) and lipoylation plasmid (ScLA). As shown in Figure 5A, after three rounds of serial transfers in SCD (LA free medium), four randomly picked up colonies were all able to grow on SCD-HIS-TRP-LEU-URA (SCD-HWLU) and the presence of the plasmids and the deletion of both *ACS1* and *ACS2* were further confirmed using diagnostic PCR (Supplementary Figure S4). Nevertheless, if the same process was carried out in LA

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supplemented medium, none of the colonies picked up could grow on the quadruple dropout medium, indicating the loss of at least one plasmid (Figure 5B).

We then tested the production of acetyl-CoA derived molecules in the engineered yeast strains, although they still grew poorer lower than the wild-type strain. When the CoA-dependent *n*-butanol biosynthetic pathway constructed in our previous work[2,26] was introduced, *n*-butanol production can be detected in all yeast strains. As shown in Supplementary Figure S5, the production of *n*-butanol was significantly lower in the DenovoLip strain, indicating that the *de novo* lipoylation pathway was not efficient enough to synthesize a sufficient amount of acetyl-CoA for cellular growth and biosynthesis. Interestingly, the production of *n*-butanol was slightly higher in the yeast strain with a scavenging lipoylation pathway, which may represent the energetic benefits of PDH over the endogenous ACS-dependent pathway for acetyl-CoA biosynthesis under anaerobic conditions.

PDH with an Unnatural *de novo* Synthetic Lipoylation Pathway. As reported in our previous studies, the reversed β-oxidation pathway could produce octanoic acid when a medium chain thioesterase gene (Cp*FatB1*) was coexpressed.[26] Therefore, as an alternative to the naturally existing *de novo* synthetic lipoylation pathway, we redesigned an unnatural *de novo* lipoylation pathway by combining the reversed β-oxidation pathway and the semisynthetic lipoylation pathway. As shown in Figure 1, octanoyl-CoA, an intermediate of the reversed β-oxidation pathway, could be thiolyzed by CpFatB1 to release octanoic acid, which could then be fed to the semisynthetic lipoylation pathway to construct a functional PDH in the cytosol of yeast. On the contrary, the control strains without either the reversed β-oxidation pathway or the lipoylation pathway showed no significant growth on synthetic, LA free medium (Figure 5C). The growth of the strain harboring this redesigned lipoylation pathway was much lower than that containing the natural *de novo* lipoylation pathway (Figure 5D), indicating that VhAasS might be rate-limiting, the reversed β-oxidation pathway was not as efficient as the type II FAS, or there was too much burden to maintain an additional multiple copy plasmid.

There is a growing interest in developing *S. cerevisiae* as a cell factory for the production of biofuels and chemicals, owing to its high tolerance to harsh industrial conditions.[27,28] Besides its essential role in numerous metabolic pathways and cellular functions, acetyl-CoA is the building block for the biosynthesis of many products of industrial interest. However, acetyl-CoA metabolism in yeast is rather complex and highly regulated. In addition, this precursor molecule is synthesized in various compartments and cannot be directly transported between these compartments. Because of the lack of a machinery to export the mitochondrial acetyl-CoA to the cytosol,[27] the activation of acetate is the only route to generate cytosolic acetyl-CoA in *S. cerevisiae*. This PDH-bypass route suffers from low metabolic fluxes, low enzyme activity, and high energy input requirement. Therefore, the introduction of heterologous synthetic pathways with higher efficiency and lower energy input requirement should be able to enhance the availability of acetyl-CoA in the cytosol. In this study, we focus on PDH, the most widely distributed and perhaps the most efficient route, for acetyl-CoA generation in the cytosol of yeast.

PDH from *E. coli* was characterized to enable the growth of the Acs$^-$ strain on glucose synthetic medium, indicating that PDH was functional to replace the endogenous ACS-dependent pathway for acetyl-CoA biosynthesis. However, the functional PDH could only be obtained when the lipoylation machinery (either the scavenging pathway with LA supplementation or the *de novo* synthetic pathway) was also included, which seemed to be contradictory to our previous results showing that the overexpression of PDH structural genes increased the cytosolic acetyl-CoA level and accordingly the production of *n*-butanol.[2] This discrepancy may be caused by the use of yeast strains with different genetic backgrounds: unlipoylated PDH might possess some minimal activity that results in more acetyl-CoA in the Acs$^+$ strain, but was not high enough to provide sufficient amount of acetyl-CoA for cell survival in the Acs$^-$ strain. Nevertheless, compared with the control strain, we consistently observed marginally improved growth in the PDH overexpressing strain in LA free medium (Figure 2B). It is also possible that the overexpression of PDH structural genes affects the acetyl-CoA metabolism, which is rather complicated and highly regulated, and increases the acetyl-CoA level in the cytosol indirectly. For example, it has been shown recently that Ach1p, whose involvement in acetyl-CoA metabolism is highly regulated, is responsible for shuttling acetyl-CoA from the mitochondria to the cytosol[29] and the expression of PDH structure genes may affect the expression and/or activity of Ach1p to change the metabolism of acetyl-CoA. Nevertheless, a clear explanation for this discrepancy is still under investigation in our laboratory. Most recently, another study reported the enhanced acetyl-CoA level and increased production of triacetic acid lactone by overexpressing the *E. coli* PDH without lipoylation in *S. cerevisiae*.[30] Since the same PDH genes were chosen and the lipoylation machinery was not included, this study further supports our hypothesis that unlipoylated PDH is active and can enhance the supply of cytosolic acetyl-CoA for biosynthesis in Acs$^+$ background yeast strain. Meanwhile, we tested the activity of PDH in another yeast strain with a different genetic background, the Pdc$^-$ strain, which is deficient in pyruvate decarboxylase activity (*pdc1*Δ *pdc5*Δ *pdc6*Δ) and evolved for growth on glucose synthetic medium.[31] Interestingly, a shift in fermentation profiles was observed: alcohol fermentation in the Pdc$^+$ strain while acid fermentation in the Pdc$^-$ strain. If an *n*-butanol biosynthetic pathway was introduced into such a strain, no *n*-butanol or butyric acid production could be detected, probably due to the limited supply of acetyl-CoA. When PDH was coexpressed, we could detect the production of a small amount of butyric acid, whose production was further increased if LA was supplemented (Supplementary Figure S6). These results also indicated that unlipoylated PDH could enhance the supply of acetyl-CoA in the Pdc$^-$Acs$^+$ strain, although the lipoylated form showed much higher activity. In terms of metabolic engineering applications, the expression of a functional PDH in the Pdc$^-$ strain, in which ethanol production was completely eliminated and pyruvate was accumulated to high levels, will channel most of the metabolic fluxes from glucose to pyruvate and then to cytosolic acetyl-CoA and hereby lay the foundation for efficient production of a wide range of value-added compounds. Unfortunately, our efforts to engineer acetyl-CoA level in the Pdc$^-$ strain led to limited success. Therefore, a systematic understanding of the metabolism of the Pdc$^-$ strain will be required for the design and construction of an acetyl-CoA overproducing yeast strain.

Considering the high cost of LA, a *de novo* synthetic lipoylation pathway should be included to construct a functional PDH in the cytosol of yeast for industrial

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applications. Through coexpression of the *E. coli* PDH structural genes, the lipoylation pathway from the mitochondria of *S. cerevisiae*, and the type II FAS genes from *E. coli* in the cytosol, the growth of the Acs$^-$ strain on glucose synthetic medium was rescued, indicating that PDH was lipoylated and functional. In addition, an unnatural *de novo* lipoylation pathway was designed and constructed by combining the semisynthetic lipoylation pathway based on acyl-ACP synthetase (VhAasS) with the reversed β-oxidation pathway. Although the growth of these engineered strains was still rather poor, compared with the wild-type yeast strain or the strain with a scavenging lipoylation pathway, protein engineering and pathway engineering strategies can be performed to optimize the performance of these pathways.[32,33] Since the growth is the direct readout of the cytosolic acetyl-CoA level, a high throughput screening method can be readily developed based on the growth rate. In addition, since octanoyl-ACP is the direct precursor for lipoylation, there would be a driving force to synthesize octanoyl-ACP by the type II FAS or octanoyl-CoA by the reversed β-oxidation pathway. Octanoyl-CoA and octanoyl-ACP are also the precursors to synthesize octanol and heptane, both of which have nearly the same fueling properties as gasoline.[34] Compared with the type I FAS in the cytosol of yeast, the reconstituted type II FAS from *E. coli* allows for more flexible product profiles.[35]

CONCLUSIONS

Acetyl-CoA biosynthetic pathway from *E. coli* (PDH together with the corresponding lipoylation machinery) was introduced into the cytosol of *S. cerevisiae* via synthetic biology approaches, which could replace the endogenous ACS-dependent pathway for acetyl-CoA biosynthesis. To construct a functional PDH in the cytosol of yeast, different lipoylation pathways were introduced. Besides the naturally existing scavenging pathway and *de novo* biosynthetic lipoylation pathway, we tested a semisynthetic lipoylation pathway based on the acyl-ACP synthetase (VhAasS), with the supplementation of octanoic acid to the growth medium. Then we designed and constructed an unnatural *de novo* lipoylation pathway, which combined the semisynthetic lipoylation pathway with the reversed β-oxidation pathway. The scavenging pathway resulted in a functional PDH that enabled the growth of the Acs$^-$ strain to a similar level of the wild-type strain, when LA was supplemented. The *de novo* lipoylation pathways led to a much lower growth rate and biomass yield, indicating a necessity of further protein engineering and pathway engineering efforts. Nevertheless, we report for the first time on the reconstitution of functional *de novo* synthetic lipoylation pathways in the cytosol of yeast, which has laid the foundation for further optimization of acetyl-CoA biosynthesis in the yeast cell factory.

METHODS

Strains, Media, and Cultivation Conditions. *E. coli* strain DH5α was used to maintain and amplify plasmids. *S. cerevisiae* CEN.PK2-1C was used as the host for homologous recombination based cloning, heterologous gene expression, and growth assays. Yeast strains were cultivated in complex medium consisting of 2% peptone and 1% yeast extract supplemented with either 2% glucose (YPD) or 2% ethanol (YPE). Recombinant strains were grown on synthetic complete (SC) medium consisting of 0.17% yeast nitrogen base, 0.5% ammonium sulfate, and 0.07% amino acid drop out mix (CSM-HIS-TRP-LEU-URA, MP Biomedicals, Solon, OH), supplemented with 2% glucose (SCD) or 2% ethanol (SCE). *E. coli* strains were cultured at 37 °C in Luria−Bertani broth containing 100 μg/mL ampicillin. *S. cerevisiae* strains were cultured at 30 °C and 250 rpm. To select for antibiotic resistant *S. cerevisiae* strains, G418 was supplemented at a final concentration of 200 μg/mL. LA and octanoic acid were dissolved in ethanol as 1000× stock solution and supplemented to the growth medium at a final concentration of 100 μg/L and 10 mg/L, respectively, unless specifically mentioned. All restriction enzymes, Q5 High Fidelity DNA polymerase, and the *E. coli*-*S. cerevisiae* shuttle vectors were purchased from New England Biolabs (NEB, Ipswich, MA). All chemicals were purchased from either Sigma-Aldrich or Fisher Scientific.

DNA Manipulation. Recombinant plasmids constructed in this study were listed in Table 1. Oligonucleotides used for gene amplification and pathway assembly were provided in Supplementary Table S1. Candidate genes were cloned into the helper plasmids constructed in our previous studies[26] using restriction-ligation or Gibson assembly, which were then assembled into multiple-gene pathways using the DNA assembler method.[36] To verify the correctly assembled pathways, yeast plasmids were isolated using a Zymoprep Yeast Plasmid Miniprep II Kit (Zymo Research, Irvine, CA) and retransformed into *E. coli* DH5α or NEB Turbo competent cells. To construct NADH insensitive PDH mutants (PDHm1 and PDHm2), mutation at the E354 site of LpdA (E354K or

Table 1. Plasmids Constructed in This Study[a]

plasmid	construct
PDHwt	pRS414-*TEF1p*-Ec*LpdA*-*PGK1t*-*TPI1p*-Ec*AceE*-*GPDt*-*ENO2p*-Ec*AceF*-*TEF1t*
PDHm1	pRS414-*TEF1p*-Ec*LpdA*^{E354K}-*PGK1t*-*TPI1p*-Ec*AceE*-*GPDt*-*ENO2p*-Ec*AceF*-*TEF1t*
PDHm2	pRS414-*TEF1p*-Ec*LpdA*^{E354G}-*PGK1t*-*TPI1p*-Ec*AceE*-*GPDt*-*ENO2p*-Ec*AceF*-*TEF1t*
BsLplJ	pH5-Bs*LplJ*
EcLplA	pH5-Ec*LplA*
M2J	PDHm2-*TEF1*p-Bs*LplJ*-*TEF1t*
BsLA	pRS425-*ENO2p*-Bs*GcvH*-*PGK1t*-*TPI1p*-Bs*LipA*-*TPI1t*-*TEF1p*-Bs*LipM*-*TEF1t*-*PGK 1p*-Bs*LipL*-*HXT7t*
EcLA	pRS425-*TPI1p*-Ec*LipA*-*TPI1t*-*TEF1p*-Ec*LipB*-*TEF1t*
ScLA	pRS425-*ENO2p*-cyto*GCV3*-*PGK1t*-*TPI1p*-cyto*LIP5*-*TPI1t*-*TEF1p*-cyto*LIP3*-*TEF1t*-*PGK1p*-cyto*LIP2*-*HXT7t*
VhEc	pRS413-*TPI1p*-Ec*AcpP*-*TPI1t*-*TEF1p*-Ec*AcpS*-*TEF1t*-*PGK1p*-Vh*AasS*-*HXT7t*
VhSc	pRS413-*TPI1p*-cyto*ACP1*-*TPI1t*-*TEF1p*-cyto*PPT2*-*TEF1t*-*PGK1p*-Vh*AasS*-*HXT7t*
rP48	pRS426-*GPM1p*-cyto*FOX3*-*ADH1t*-*GPDp*-cytoYl*KR*-*CYC1t*-*ENO2p*-cyto*ETR1*-*PGK1t*-*TEF1p*-Cp*FatB1*-*TEF1t*-*PGK1p*-cytoYl*HTD*-*HXT7t*
cytoFAA2	pRS423-*TEF1p*-cyto*FAA2*-*TEF1t*
cytoFAS0	pRS413-*GPM1p*-cyto*CEM1*-*ADH1t*-*PYK1p*-cyto*OAR1*-*CYC1t*-*ENO2p*-cyto*ETR1*-*PGK1t*-*TPI1p*-cyto*ACP1*-*TPI1t*-*TEF1p*-cyto*PPT2*-*TEF1t*-*PGK1p*-cyto*MCT1*-*HXT7t*
cytoFAS1	cytoFAS0-*HXT7p*-cyto*HTD2*-*HXT7t*
cytoFAS2	cytoFAS0-*HXT7p*-*EcFabA*-*HXT7t*
cytoFAS3	cytoFAS0-*HXT7p*-*EcFabZ*-*HXT7t*
EcFAS0	pRS413-*GPM1p*-Ec*FabD*-*ADH1t*-*PYK1p*-Ec*FabG*-*CYC1t*-*ENO2p*-Ec*FabI*-*PGK1t*-*TPI1p*-Ec*AcpP*-*TPI1t*-*TEF1p*-Ec*AcpS*-*TEF1t*-*PGK1p*-Ec*FabF*-*HXT7t*
EcFAS0A	EcFAS0-*HXT7p*-*EcFabA*-*HXT7t*
EcFAS	EcFAS0A-*ENO2p*-*EcFabH*-*PGK1t*

[a]Bs, *Bacillus subtilis*; Ec, *Escherichia coli*; Vh, *Vibrio harveyi*; cyto, cytosol relocalization by removing the targeting signal sequences.

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E354G) was introduced by overlapped extension PCR and the resultant DNA fragment was cloned into the *Afe*I and *Pac*I sites of PDHwt. To construct M2J, Bs*LplJ* cassette (*TEF1p*-Bs*LplJ*-*TEF1t*) was cloned into the *Sal*I site of PDHm2 by the Gibson assembly method. To construct cytoFASs, the mitochondrial targeting sequences of the candidate proteins were mainly predicted by MITOPROT (http://ihg.gsf.de/ihg/mitoprot.html)[37] and excluded during the cloning process (Supplementary Table S2). CytoFAS0 was constructed first using the helper plasmid based DNA assembler method, and then the *HTD2* cassette (*HXT7p-cytoHTD2-HXT7t*), the Ec*FabA* cassette (*HXT7p*-Ec*FabA-HXT7t*), and the Ec*FabZ* cassette (*HXT7p*-Ec*FabZ-HXT7t*) were cloned into the *Stu*I site of cytoFAS0 to construct cytoFAS1, cytoFAS2, and cytoFAS3, respectively. To construct the *E. coli* type II FAS, EcFAS0 was constructed first using the DNA assembler method, and then the Ec*FabA* cassette (*HXT7p*-Ec*FabA-HXT7t*) was cloned into the *Stu*I site to construct EcFAS0A, followed by the introduction of the Ec*FabH* cassette (*ENO2p*-Ec*FabH*-*PGK1t*) into the *Not*I site to construct EcFAS. The reversed β-oxidation pathway rP48 was constructed by replacing Ec*EutE*-Ca*BdhB* cassettes in rP35[26] with Cp*FatB1* expression cassette (*TEF1p*-Cp*FatB1*-*TEF1t*). Plasmids were isolated using a QIAprep Spin Miniprep Kit (Qiagen) and confirmed using both diagnostic PCRs and restriction digestions (Supplementary Figure S7). Yeast strains were transformed using the LiAc/SS carrier DNA/PEG method,[38] and transformants were selected on the appropriate SC plates.

Strain Construction. All the strains used in this study were based on CEN.PK2-1C and are listed in Table 2. The *Cre-loxP* method[39] was used to construct all knockout strains. For the construction of the *acs2*Δ strain, the *loxP*-Kl*URA3-loxP* cassette was PCR amplified with pUG72 as the template to include 40 bp homology arms at both ends, and transformed into CEN.PK2-1C strain. The transformants were screened on SCE-URA plates and the resultant *acs2*Δ strain was maintained in medium with ethanol as the sole carbon source. After restoring the growth on glucose, *ACS1* was further deleted by transforming the *loxP-KanMX-loxP* cassette amplified from pUG6 and selection on YPD plates supplemented with 200 mg/L G418. The resultant Acs$^-$ strains (*acs1*Δ *acs2*Δ) containing M2J were maintained on glucose medium supplemented with LA and used as an *in vivo* reporter of acetyl-CoA level in the cytosol. The selection markers in the engineered strains were rescued by transforming the *Cre* expressing plasmid (pSH63).

Table 2. Strains Used in This Study

strain	genotype
CEN.PK2-1C	*MATa ura3−52 trp1−289 leu2−3,112 his3Δ1 MAL2−8^C SUC2*
*acs2*Δ	CEN.PK2-1C *acs2*Δ::*URA3*
*acs2*Δ/wtBs	CEN.PK2-1C *acs2*Δ::*URA3*/PDHwt+BsLplJ
*acs2*Δ/wtEc	CEN.PK2-1C *acs2*Δ::*URA3*/PDHwt+EcLplA
*acs2*Δ/m1Bs	CEN.PK2-1C *acs2*Δ::*URA3*/PDHm1+BsLplJ
*acs2*Δ/m2Bs	CEN.PK2-1C *acs2*Δ::*URA3*/PDHm2+BsLplJ
Acs$^-$/M2J	CEN.PK2-1C *acs2*Δ::*URA3 acs1*Δ::*KanMX*/M2J
Acs$^-$K/M2J	CEN.PK2-1C *acs2*Δ::*loxP acs1*Δ::*KanMX*/M2J
SemiLip	Acs$^-$/M2J+ScLA+VhEc
DenovoLip	Acs$^-$/M2J+ScLA+EcFAS
ReLipNC1	Acs$^-$K/M2J+ScLA+VhSc+pRS426
ReLipNC2	Acs$^-$K/M2J+pRS425+VhSc+rP48
ReLip	Acs$^-$K/M2J+ScLA+VhSc+rP48
Pdc$^-$[31]	CEN.PK2-1C *pdc5*Δ::*loxP pdc6*Δ::*loxP pdc1*Δ::*KanMX*, evolved for growth on glucose

Growth Assays. The *acs2*Δ yeast strains were pregrown in the appropriate synthetic medium with 2% ethanol as the carbon source for about 5 days. The Acs$^-$/M2J strain was pregrown in glucose synthetic medium supplemented with 100 μg/L LA. Then yeast cells were collected by centrifugation, washed three times in sterile ddH_2O, and cells with appropriate dilutions (10^4, 10^3, 10^2, and 10^1 cells, assuming 10^7 cells/mL equivalent to OD_{600} of 1.0) were spotted into synthetic medium agar plates containing 2% ethanol, 2% glucose, and 2% glucose supplemented with 100 μg/L LA, respectively. Strains on glucose with LA plates were incubated at 30 °C for 3 days, while those on ethanol and glucose plates were incubated at 30 °C for 5 days. Liquid growth assays were performed using a BioScreen C (Growth Curves USA, Piscataway, NJ) in four replicates. Fully grown seeds were inoculated into 200 μL of the corresponding synthetic medium with an initial OD around 0.1. Tween80 or Brij58 was supplemented at a final concentration of 0.05%. The experiment was run at 30 °C for 5 days with continuous shaking and sampling every 1 h. To eliminate the effect of trace amount of LA on the growth of the Acs$^-$, PDH expressing strains, LA was drained by transferring the yeast cells to fresh SCD liquid medium or agar plate for at least three times, or until completely no cell growth was observed for the control strains.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.6b00019.

Additional figures and tables as described in the text (PDF)

■ AUTHOR INFORMATION

Corresponding Author

*Tel.: (217) 333-2631. Fax: (217) 333-5052. E-mail: zhao5@illinois.edu.

Author Contributions

J.L. and H.Z. designed and analyzed experiments, J.L. conducted experiments, and J.L. and H.Z. wrote the manuscript.

Notes

The authors declare no competing financial interest.

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