Engineering Acetyl Coenzyme A Supply: Functional Expression of a Bacterial Pyruvate Dehydrogenase Complex in the Cytosol of Saccharomyces cerevisiae

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ABSTRACT The energetic (ATP) cost of biochemical pathways critically determines the maximum yield of metabolites of vital or commercial relevance. Cytosolic acetyl coenzyme A (acetyl-CoA) is a key precursor for biosynthesis in eukaryotes and for many industrially relevant product pathways that have been introduced into Saccharomyces cerevisiae, such as isoprenoids or lipids. In this yeast, synthesis of cytosolic acetyl-CoA via acetyl-CoA synthetase (ACS) involves hydrolysis of ATP to AMP and pyrophosphate. Here, we demonstrate that expression and assembly in the yeast cytosol of an ATP-independent pyruvate dehydrogenase complex (PDH) from Enterococcus faecalis can fully replace the ACS-dependent pathway for cytosolic acetyl-CoA synthesis. In vivo activity of E. faecalis PDH required simultaneous expression of E. faecalis genes encoding its E1α, E1β, E2, and E3 subunits, as well as genes involved in lipoylation of E2, and addition of lipoate to growth media. A strain lacking ACS that expressed these E. faecalis genes grew at near-wild-type rates on glucose synthetic medium supplemented with lipoate, under aerobic and anaerobic conditions. A physiological comparison of the engineered strain and an isogenic Acsc− reference strain showed small differences in biomass yields and metabolic fluxes. Cellular fractionation and gel filtration studies revealed that the E. faecalis PDH subunits were assembled in the yeast cytosol, with a subunit ratio and enzyme activity similar to values reported for PDH purified from E. faecalis. This study indicates that cytosolic expression and assembly of PDH in eukaryotic industrial microorganisms is a promising option for minimizing the energy costs of precursor supply in acetyl-CoA-dependent product pathways.

IMPORTANCE Genetically engineered microorganisms are intensively investigated and applied for production of biofuels and chemicals from renewable sugars. To make such processes economically and environmentally sustainable, the energy (ATP) costs for product formation from sugar must be minimized. Here, we focus on an important ATP-requiring process in baker’s yeast (Saccharomyces cerevisiae): synthesis of cytosolic acetyl coenzyme A, a key precursor for many industrially important products, ranging from biofuels to fragrances. We demonstrate that pyruvate dehydrogenase from the bacterium Enterococcus faecalis, a huge enzyme complex with a size similar to that of a ribosome, can be functionally expressed and assembled in the cytosol of baker’s yeast. Moreover, we show that this ATP-independent mechanism for cytosolic acetyl-CoA synthesis can entirely replace the ATP-costly native yeast pathway. This work provides metabolic engineers with a new option to optimize the performance of baker’s yeast as a “cell factory” for sustainable production of fuels and chemicals.
synthetic product pathways in robust industrial microorganisms is being increasingly explored for production of valuable compounds from renewable feedstocks. Fast developments in yeast synthetic biology and systems biology, as well as its robustness, have made *Saccharomyces cerevisiae* one of the most popular metabolomic engineering platforms in modern biotechnology (11). However, efficient provision of acetyl-CoA in this yeast presents formidable challenges.

As for all eukaryotic PDHs, the *S. cerevisiae* PDH is located in the mitochondrial matrix. However, in this yeast, acetyl-CoA generated in the mitochondrion cannot meet the requirement for cytosolic acetyl-CoA. Instead, a separate pathway known as the pyruvate dehydrogenase bypass, which involves pyruvate decarboxylase, acetaldehyde dehydrogenase, and acetyl-CoA synthetase, provides cytosolic acetyl-CoA for essential biosynthetic processes, such as the production of lipids, lysine, and sterols (12). Since intracellular transport of products and intermediates across mitochondrial membranes is difficult to engineer, heterologous product pathways are generally expressed in the yeast cytosol (but see reference 13). Productivities and yields of acetyl-CoA dependent products in engineered *S. cerevisiae* strains have been shown to improve upon increasing the capacity of the native *S. cerevisiae* pathway or expressing heterologous pathways for cytosolic acetyl-CoA synthesis (8, 14–17).

A problem that is even more challenging than pathway capacity concerns the energetic costs of cytosolic acetyl-CoA synthesis. The acetyl-CoA synthetase reaction involved in the native *S. cerevisiae* pathway for cytosolic acetyl-CoA synthesis includes hydrolysis of ATP to AMP and pyrophosphate. Subsequent hydrolysis of pyrophosphate to inorganic phosphate makes the formation of AMP from ATP energetically equivalent to hydrolysis of 2 ATP molecules to 2 ADP and 2 inorganic phosphate molecules. This ATP cost for synthesis of cytosolic acetyl-CoA has a huge impact on the maximum yield of acetyl-CoA-derived products from feedstocks and, consequently, on the economic and environmental sustainability of yeast-based processes. For example, it has been estimated that in *S. cerevisiae*, an extra mole of glucose (180 g) has to be respired to carbon dioxide and water just to meet the ATP requirement for cytosolic acetyl-CoA synthesis in the production of 1 mol of a C6 lipid (e.g., palmitic acid, 256 g). Especially for production of bulk chemicals and fuels, such an ATP expenditure is simply not compatible with process economy. In a recent study, the native pathway for synthesis of cytosolic acetyl-CoA in *S. cerevisiae* was replaced by ATP-neutral pathways involving either acetylating acetaldehyde dehydrogenase (AALD) or pyruvate-formate lyase (PFL). Although these genetic modifications were able to complement a double deletion of the two *S. cerevisiae* genes encoding acetyl-CoA synthetase, biomass yields were lower than in the parental strain, probably due to the accumulation of toxic by-products (18).

The goal of this study was to determine whether PDH can supply an ATP-independent pathway for cytosolic acetyl-CoA synthesis, by using functional expression and *in vivo* assembly of a bacterial PDH in the *S. cerevisiae* cytosol. To this end, codon-optimized genes encoding the E1α, E1β, E2, and E3 subunits of the *Enterococcus faecalis* PDH were expressed in *S. cerevisiae*, together with *E. faecalis* genes encoding proteins involved in lipoylation of E2. Expression of the PDH subunits, their cytosolic localization, and *in vivo* assembly into a PDH complex were analyzed by mass spectrometry, subcellular fractionation, and gel filtration, respectively. Enzymatic activity assays demonstrated that the heterologous cytosolic PDH complex was more active than the native mitochondrial PDH complex in the engineered strains. The consequences of replacing the native *S. cerevisiae* pathway by a cytosolically expressed heterologous PDH on physiology and the transcriptome were investigated in chemostat cultures.

**RESULTS**

Expression of *Enterococcus faecalis* pyruvate dehydrogenase complements deletion of acetyl-CoA synthetase in the presence of lipoic acid. To determine if PDH can replace the native cytosolic route to acetyl-CoA, the native route must be blocked (e.g., by deletion of ACS genes) and the subunits of PDH must be expressed such that they will not be targeted to the mitochondrion (as is the case for the native PDH complex). Three factors favored the choice of the PDH from *Enterococcus faecalis*: bacterial PDH subunits presumably have no mitochondrial localization sequences; *E. faecalis* PDH is relatively insensitive to high NADH/NAD+ ratios, which allows it to function under anaerobic as well as aerobic conditions in its natural host; the *E. faecalis* PDH complex has been shown to self-assemble from purified components in *vitro* (19, 20). The second characteristic may be advantageous in metabolic engineering of *S. cerevisiae*, in which cytosolic NADH/NAD+ ratios are strongly condition dependent (21).

The activity of PDH depends on lipoylation of the E2 subunit (22, 23). Based on genome annotation and similarity with genes encoding ligases involved in protein lipoylation, *E. faecalis* lplA and lplA2 were identified as putative lipoylation genes. LplA and LplA2 showed 43% and 58% similarity, respectively, with the lipoyte-protein ligase LplJ of *Bacillus subtilis* (24). Codon-optimized genes encoding the E1α, E1β, E2, and E3 subunits of *Enterococcus faecalis* PDH encoded by pUDEhA, pUDEb, aceF, and lpld, as well as codon-optimized lplA and lplA2 genes, were expressed in *S. cerevisiae* lacking ACS activity to eliminate the native cytosolic route to acetyl-CoA.

In *S. cerevisiae*, ACS1 and ACS2 encode isoenzymes of acetyl-CoA synthetase (25, 26). In the presence of glucose, ACS1 transcript is repressed and the Acsl protein is inactivated; thus, acs2Δ mutants cannot grow on glucose plates (27, 28). Since Acsl is active during growth on ethanol, acs2Δ strains can be pregrown on ethanol. This phenotype of the acs2Δ strain IMK427 was used in initial tests for functional expression of the *E. faecalis* PDH complex in *S. cerevisiae*. Plasmids carrying the four *E. faecalis* PDH genes and the two lipoylation genes were assembled by *in vivo* homologous recombination into *S. cerevisiae* IMK427. To investigate the impact of copy number, the *E. faecalis* genes were expressed from both high-copy-number (2µ) and low-copy-number (centromeric) plasmids, yielding strains IMZ466 (acs2Δ pUDE333; 2µ vector with expression cassettes for pdhA, pdhB, aceF, lpld, lplA, and lplA2) and IMY102 (acs2Δ pUDC140; centromeric vector with expression cassettes for pdhA, pdhB, aceF, lpld, lplA, and lplA2) and IMY109 (acs2Δ pUDC143; centromeric vector with expression cassettes for pdhA, pdhB, aceF, and lpld but not lplA and lplA2) was constructed.

In the absence of lipoic acid, only the reference strain CEN.PK113-7D (ACS2) grew on synthetic medium agar plates containing 20 g liter⁻¹ glucose (Fig. 1), indicating that, under these conditions, the expressed *E. faecalis* PDH could not provide sufficient cytosolic acetyl-CoA to complement the ACS2 deletion.
However, when lipoic acid was included in the growth medium, strains IMZ466 and IMY102, which expressed the E. faecalis PDH subunits and lipoylation genes, did grow on glucose. Under the same conditions, neither IMK427 (acs2Δ) nor IMY104 (acs1Δ, expressing the E. faecalis PDH subunits but not the lipoylation genes) grew, with or without lipoic acid addition (Fig. 1). In shake flask cultures grown on synthetic medium with 20 g liter⁻¹ glucose and 50 ng ml⁻¹ lipoic acid, strain IMZ466 (expressing E. faecalis PDH subunits and lipoylation genes from a multicopy plasmid) showed a longer lag phase and a lower specific growth rate (0.18 h⁻¹) than strain IMY102 (0.36 h⁻¹), in which PDH and lipoylation genes were expressed from a centromeric vector.

Although ACS1 is not expressed during growth on glucose, mutations can activate this gene during long-term cultivation on glucose medium (18). To increase stability of the PDH-expressing strains after the initial screening, ACS1 was deleted in strain IMY102 (acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2), resulting in strain IMY104 (acs1Δ,acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2). The acetyl-CoA synthase activity in cell extracts of strain IMY104 was below the detection limit of the assay (Table 1). This strain retained the ability to grow on agar plates with glucose and lipoic acid (Fig. 1). In batch cultures on synthetic medium with glucose as carbon source, supplemented with 50 ng ml⁻¹ lipoic acid, strain IMY104 grew at a specific growth rate of 0.35 h⁻¹. Under the same conditions, the specific growth rate of the reference strain CEN.PK113-7D (ACS1 ACS2) was 0.42 h⁻¹. Neither IMY104 nor CEN.PK113-7D showed significant changes in specific growth rate when the lipoic acid concentration was increased to 1,000 ng ml⁻¹. However, final optical densities reached in cultures of strain IMY104 (acs1Δ,acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2), but not in cultures of the reference strain CEN.PK113-7D, increased with increasing concentrations of lipoic acid (Fig. 2). This suggests that this cofactor may be depleted toward the end of the shake flask cultivations with lower lipoic acid concentrations. The IMY104 strain was also able to grow under anaerobic conditions. In anaerobic batch cultures with glucose as carbon source, supplemented with 50 ng ml⁻¹ lipoic acid, strain IMY104 (acs1Δ,acs2Δ

### TABLE 1 PDH and ACS activities of Saccharomyces cerevisiae reference strain CEN.PK113-7D and strains expressing subunits of the PDH complex from Enterococcus faecalis

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>Enzyme sp act (nmol/min/mg of protein)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>PDH</td>
</tr>
<tr>
<td>CEN.PK113-7D</td>
<td>ACS1 ACS2 PDC1 PDC5 PDC6 PDA1</td>
<td>12 ± 2</td>
</tr>
<tr>
<td>IMY104</td>
<td>acs1Δ,acs2Δ pUDC140;CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2</td>
<td>53 ± 2</td>
</tr>
<tr>
<td>IMX216</td>
<td>pdc1Δ pdc3Δ pdc6Δ pda1Δ MTH1-ΔT</td>
<td>BDL</td>
</tr>
<tr>
<td>IMU064</td>
<td>pdc1Δ pdc3Δ pdc6Δ pda1Δ MTH1-ΔT pUDE333;2µ pdhA pdhB aceF lpd lplA lplA2</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>IMQ011</td>
<td>pdc1Δ pdc3Δ pdc6Δ pda1Δ MTH1-ΔT pUDE3140;CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2</td>
<td>30 ± 1</td>
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</table>

*All strains were pregrown in shake flasks with glucose, for strains CEN.PK113-7D and IMY104, or ethanol, for strains IMX216, IMU064, and IMQ011, as a carbon source, supplemented with 50 ng ml⁻¹ lipoic acid. Averages and standard deviations were obtained from duplicate experiments. The detection limit of the enzyme assays was 3 nmol/min/mg of protein. BDL, below detection limit; ND, not determined.
FIG 2 Growth curves and final optical densities of cultures of *S. cerevisiae* strains MY104 (acs1Δ acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2) and CEN.PK113-7D (ACS1 ACS2) grown on synthetic medium with 20 g liter−1 glucose, supplemented with different concentrations of lipoic acid. Strain CEN.PK113-7D had a specific growth rate of 0.42 h−1. The growth rate of the IMU064 strain was independent of lipoic acid concentration and equal to 0.35 h−1. The single experiment was qualitatively representative of duplicate experiments. Symbols for IMY104 results: ■, 20 ng ml−1 lipoic acid; △, 50 ng ml−1 lipoic acid; ▼, 100 ng ml−1 lipoic acid; ●, 200 ng ml−1 lipoic acid; ○, 500 ng ml−1 lipoic acid; ◆, 1,000 ng ml−1 lipoic acid; ○, CEN.PK113-7D (with 20 ng ml−1 lipoic acid).

pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2 grew at a specific growth rate of 0.30 h−1. Under the same conditions, the specific growth rate of the reference strain CEN.PK113-7D (ACS1 ACS2) was 0.33 h−1.

**Cytosolic expression of the *E. faecalis* PDH in yeast.** Coexpression of the four subunits of the *E. faecalis* PDH complex together with *lplA* and *lplA2* enabled growth of *Acs−* *S. cerevisiae* on glucose in the presence of externally added lipoic acid. To further investigate the expression of the PDH subunits, their subcellular localization and in vivo assembly were analyzed by *in vitro* measurements of enzyme activity, mass spectrometry, subcellular fractionation, and gel filtration, respectively.

The enzymatic activities of native and introduced PDH in the reference strain CEN.PK113-7D and in IMY104 (acs1Δ acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2) were measured in cell extracts. The activity of the native PDH in the wild-type strain was 12 ± 2 nmol min−1 (mg of protein)−1 (mean ± standard deviation), while the PDH activity measured in the IMY104 strain, which represents the combined activities of the native and introduced PDH complexes, was significantly higher, at 53 ± 2 nmol min−1 (mg of protein)−1. In the IMX216 host strain (*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1−ΔT*), PDH activity was below the detection limit (Table 1). Expression of *E. faecalis* PDH and lipoylation genes in strains IMU064 (*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1−ΔT* pUDE333; 2 μg *pda1Δ pdhA aceF* lpd lplA lplA2) and IMQ011 (*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1−ΔT* pUDE140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2) yielded enzyme activities of 18 ± 1 and 30 ± 1 nmol min−1 (mg of protein)−1, respectively. These results are in accordance with the observation that strains carrying PDH on multicopy plasmids grew slower than strains with PDH on low-copy-number plasmids.

The cytosolic localization of the introduced PDH complex is essential for both the replacement of the native pathway of cytosolic acetyl-CoA synthesis and envisioned industrial applications.

To check the presence of the introduced *E. faecalis* PDH complex in the cytosol of *S. cerevisiae* and distinguish its activity from that of the native mitochondrial PDH complex, the cytosolic and mitochondrial fractions of IMY104 (acs1Δ acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2) and CEN.PK113-7D (ACS1 ACS2). White bars, IMY104; black bars, CEN.PK113-7D. Averages and standard deviations were obtained from two replicate experiments.

To check whether the introduced PDH was present in the cytosol of yeast as individual subunits or as a complex, size exclusion chromatography was used. PDH activity was observed in 13 sequential fractions, starting from fraction 14 (out of 50 fractions collected), in which a peak specific activity of 8.8 μmol min−1 (mg of protein)−1 was measured (Fig. 4). This activity was similar to a
previously reported activity for purified PDH complex from *E. faecalis* of 7.8 μmol min⁻¹ (mg of protein)⁻¹ (20). Comparison of the specific activities of PDH in the homogenate of IMY104, which was 0.067 μmol min⁻¹ (mg of protein)⁻¹, and in fraction 14 obtained from the chromatographic column, was 8.8 μmol min⁻¹ (mg of protein)⁻¹, indicated a >130-fold purification of the PDH complex (Table 2).

To further investigate the presence of PDH as a complex in the cytosol of strain IMY104, mass spectrometry-based proteomics was applied to the first 8 column fractions in which PDH activity was detected. In each of the analyzed samples, all subunits of the PDH complex were identified. The relative abundance of subunits E1, E2, and E3 (2.27 ± 0.19:1 ± 0.10:1.26 ± 0.13; mass ratio, averaged from the first three fractions) obtained from analysis of the fractions was similar to that reported (2.05:1:0.95) for purified PDH complex in *E. faecalis* (20). The majority of the impurities identified by the proteomics in analyzed fractions consisted of ribosomal proteins. The level of those impurities increased in each subsequent fraction, which indicates that the size of the *E. faecalis* PDH complex purified on the column was likely larger than the size of the yeast ribosomes present in the soluble fraction that was applied to the column. Additionally, the proteomics analysis showed that the conserved lysine of the E2 subunit was lipoylated in the purified PDH complex (data not shown), which further confirmed the functional expression of *lplA* and *lplA2*.

Efficient growth and metabolism of Acs− *S. cerevisiae* through cytosolic expression of *E. faecalis* PDH. Replacement of the native route to cytosolic acetyl-CoA might have substantial effects on cellular physiology, by perturbing synthesis of lipids and altering regulatory acetylation of histones and many other proteins (30–34). The influence of replacing the native *S. cerevisiae* pathway for cytosolic acetyl-CoA synthesis by the *E. faecalis* PDH on quantitative physiology and transcriptome was further investigated in aerobic, glucose-limited chemostat cultures grown at a dilution rate of 0.10 h⁻¹. To avoid lipoic acid limitation, the concentration of this compound in the medium was kept at 500 μg/liter. The near-identical biomass yields on glucose of IMY104 and the reference strain, as well as the absence of significant changes in the production and the consumption rates of metabolites (Table 3), indicated that the replacement of the native route of cytosolic acetyl-CoA synthesis with the PDH complex from *E. faecalis* did not significantly influence the metabolism of *S. cerevisiae*. This was further corroborated by the absence of the strong transcriptional changes previously found after replacing the native cytosolic acetyl-CoA synthesis pathway with a heterologous acetylating acetaldehyde dehydrogenase (18). Moreover, transcriptome sequencing (RNA-seq) analysis did not show the transcriptional changes to the global histone deacetylation previously observed in response to interruption of acetyl-CoA supply (35). The chemostat-based RNA-seq analysis of CEN.PK113-7D

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**TABLE 2** Purification of the PDH complex of *E. faecalis* expressed in *S. cerevisiae*<sup>a</sup>

<table>
<thead>
<tr>
<th>Sample</th>
<th>Purification step</th>
<th>Sp act (μmol/min/mg of protein)</th>
<th>Purification factor</th>
</tr>
</thead>
<tbody>
<tr>
<td>Homogenate</td>
<td></td>
<td>0.067 ± 0.003</td>
<td>1</td>
</tr>
<tr>
<td>Cytosol C1</td>
<td>Centrifugation</td>
<td>0.062 ± 0.001</td>
<td>0.9</td>
</tr>
<tr>
<td>Cytosol C2</td>
<td>Centrifugation</td>
<td>0.071 ± 0.003</td>
<td>1.1</td>
</tr>
<tr>
<td>Cytosol C3</td>
<td>Concentration on 100-kDa cutoff filter</td>
<td>0.083 ± 0.003</td>
<td>1.2</td>
</tr>
<tr>
<td>Column fraction 14</td>
<td>Column separation</td>
<td>8.8 ± 0.25</td>
<td>131</td>
</tr>
</tbody>
</table>

<sup>a</sup> PDH activity was measured in homogenates of strain IMY104. In the next step, mitochondrial and cytosolic fractions of the homogenate were separated. The cytosolic fraction was further purified and applied onto a size exclusion chromatographic column. Fraction 14 obtained from the column showed the highest specific activity of the PDH complex. Means and standard deviations were obtained from duplicate measurements on a single column. An independent duplicate experiment gave similar results.
(ACS1 ACS2) and IMY104 (acsIΔ acs2Δ pUDP140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2) identified 88 genes whose expression levels were different in these two strains, based on the statistical criteria applied in this study (fold change of $\geq 2; P \leq 5.0 \times 10^{-5}$). The expression level of most of these genes remained low, with the notable exception of CIT2, which encodes an extramitochondrial citrate synthase. Its 4-fold upregulation in the PDH-expressing strain may reflect a cellular mechanism to feed excess of cytosolic acetyl-CoA into the TCA cycle when its production exceeds demands for biosynthesis. The RNA-seq analysis showed that all the genes coding for the PDH of $E. faecalis$—$pdhA$, $pdhB$, $aceF$, and $lpl$—were expressed at higher levels (fold change, $>2$) than the constitutively expressed ACT1, which encodes actin and is a commonly used reference gene in yeast transcript analysis (36). Expression levels of genes encoding lipoylation proteins—$lplA$ and $lplA2$—were similar to those of ACT1. Expression levels of ACT1 did not differ significantly between IMY104 and CEN.PK113-7D. The RNA-seq results also confirmed the sequence of the introduced heterologous genes.

**DISCUSSION**

Functional expression of a pyruvate dehydrogenase complex in the yeast cytosol. In this study, we demonstrate, for the first time, the functional expression, assembly, and in vivo activity of a pyruvate dehydrogenase complex in the cytosol of a eukaryotic organism. Activity of the $E. faecalis$ PDH complex in the cytosol of $S. cerevisiae$ required the expression of genes involved in lipoylation, as well as the addition of lipoate to the growth medium. The need for coexpression of lipoylation genes is not surprising, since the native $S. cerevisiae$ Lip2, Lip3, and Lip5 proteins involved in lipoylation of the E2 subunit of the yeast PDH complex are located in the mitochondrion (23, 37, 38). In vivo activity of $E. faecalis$ PDH in $S. cerevisiae$ required addition of lipoic acid to the medin, which was presumably used by the lipoate ligases for lipoylation of the cytosically expressed PDH complex. Apparently, lipoate synthesized in the mitochondrion cannot readily leave this compartment, and/or free lipoate concentrations in yeast cells are very low. Scarcity of free lipoate in yeast cells is consistent with the observation that a complex of acyl carrier protein with lipoate, rather than free lipoate, is the donor for lipoylation of E2 in yeast mitochondria (39). A recent study reported that expression of the subunits of the $E. coli$ and $S. cerevisiae$ PDH complexes in engineered $S. cerevisiae$ led to increased production of n-butanol derived from cytosolic acetyl-CoA. However, those authors did not present any data on localization, activity, or assembly of these PDH complexes, and they did not include coexpression of lipoylation genes or add lipoate to the growth medium, thereby reducing its activity under anaerobic growth conditions (19, 43). The $Acs^-$ strain expressing $E. faecalis$ PDH, however, also showed a near-wild-type specific growth rate in anaerobic cultures. This is consistent with the observation that the $E. faecalis$ PDH is much less sensitive to NADH/NAD$^+$ ratios, thereby reducing its activity under anaerobic conditions may be an important asset for use of the $E. faecalis$ PDH complex in yeast metabolic engineering studies aimed at anabolic product pathways.

Acetyl-CoA synthesis through PDH and implications for yeast metabolic engineering. The kinetics and ATP stoichiometry of cytosolic acetyl-CoA synthesis are key determinants for efficient production of many industrially relevant products. The demonstration that $E. faecalis$ PDH can be functionally expressed in $S. cerevisiae$ expands the options for metabolic engineering strategies aimed at optimizing energetics of cytosolic acetyl-CoA synthesis in this yeast. When glucose is used as the carbon source, the net ATP cost of the native pathway of cytosolic acetyl-CoA synthesis in $S. cerevisiae$ equals 1 ATP per one molecule of acetyl-CoA. The combined actions of xylulose-5-phosphate phosphoketolase (PK) and phosphate acetyltransferase (PTA) result in a cost of 0.5 ATP per molecule of acetyl-CoA, when xylulose-5-phosphate is formed from glucose-6-phosphate through the pentose phosphate pathway, and, moreover, the glyceraldehyde-3-phosphate that is produced in the reaction catalyzed by phosphoketolase is converted to acetyl-CoA through glycolysis and the native yeast pathway for acetyl-CoA synthesis (44). However, when xylulose-5-phosphate is produced from fructose-6-phosphate and glyceraldehyde-3-phosphate via the nonoxidative reactions of the pentose phosphate pathway and, moreover, the glyceraldehyde-3-phosphate that is produced in the reaction catalyzed by phosphoketolase is recycled to produce fructose-6-phosphate, the ATP cost drops to 0.4 mol/mol of acetyl-CoA. The other previously reported pathway, which involves the heterologous expression of ACL-citrate lyase (ACL) (17), results in a zero net ATP yield (Table 4). Two alternative strategies, based on the expression of heterologous acetylating acetaldehyde dehydrogenase or pyruvate-formate lyase, resulted in a decreased biomass yield compared to the $Acs^-$ reference strain, probably as a result of the accumulation of intermediates or by-

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**Figure Caption**: The figure illustrates the functional expression of a pyruvate dehydrogenase complex in the yeast cytosol. The complex is depicted as a large protein complex with a size similar to that of the ribosome, and it was not at all clear that it would be possible to reconstitute it in a heterologous host and a new subcellular compartment. Depending on the organism, assembly of PDH complexes may occur by spontaneous association of their subunits (2, 41) or require involvement of additional proteins (4, 39). In $E. faecalis$, PDH occurs as a protein complex consisting of 210 subunits with a combined estimated mass of 14 MDa (20, 42). In vitro subunit reassociation studies indicate that no additional proteins are required for assembly (20), which made $E. faecalis$ PDH an attractive candidate for expression in yeast. In the current report, gel filtration combined with enzyme activity assays and proteomics analysis demonstrated that size, specific activity, and relative abundance of the E1, E2, and E3 subunits of the heterologously expressed $E. faecalis$ PDH were consistent with those reported for native $E. faecalis$ PDH (20). To the best of our knowledge, our results represent the first successful heterologous expression of a protein complex of this size.
products\(^a\). The absence of a clear phenotype of PDH-dependent \textit{S. cerevisiae} indicates that cytosolic expression of \textit{E. faecalis} PDH does not have a similar negative impact on cellular physiology.

The present study represents a proof of principle for introduction of a heterologous PDH complex as the sole source of cytosolic acetyl-CoA in \textit{S. cerevisiae}. From a scientific point of view, it would be interesting to further engineer the PDH-dependent strains for lipoic acid prototrophy. However, addition of lipoic acid to industrial growth media at very low concentrations required by strains expressing \textit{E. faecalis} PDH should not be cost prohibitive. Assum- ing a required concentration of lipoic acid in the fermentation broth of 500 ng liter\(^{-1}\) and an approximate price below $500 per kg, the cost of added lipoate would be smaller than 25 cents per cubic meter. Further research should focus on the integration of cytosolically expressed PDH in strains containing engineered product pathways, to assess if PDH can increase specific productivity or yield of desired products and thereby reduce the costs of production.

**MATERIALS AND METHODS**

**Strains and maintenance.** The \textit{S. cerevisiae} strains used in this study (Table 5) share the CEN.PK genetic background (45, 46). Stock cultures were grown aerobically in synthetic medium (47). Auxotrophic requirements were complemented with synthetic yeast dropout medium supplements (Sigma-Aldrich, St. Louis, MO) leucine or uracil (48) or by growth in YP medium (demineralized water, 10 g liter\(^{-1}\) Bacto yeast extract, 20 g liter\(^{-1}\) Bacto peptone). When required, lipoic acid (Sigma-Aldrich) was added to the medium at the concentration of 50 mg mol\(^{-1}\). Carbon sources were either 20 g liter\(^{-1}\) glucose or 2% (vol/vol) ethanol. Frozen stocks of \textit{S. cerevisiae} and \textit{E. coli} were prepared by the addition of glycerol (30% [vol/vol]) to the growing shake flask cultures and aseptically stored in 1-ml aliquots at \(-80^\circ\text{C}\).

**TABLE 4** Energetics of different engineering approaches for improved acetyl-CoA synthesis

<table>
<thead>
<tr>
<th>Pathway</th>
<th>Molar ratio</th>
<th>Reference</th>
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<tbody>
<tr>
<td>PDH</td>
<td>ATP/acetyl-CoA</td>
<td>NADH/acetyl-CoA</td>
</tr>
<tr>
<td>A-ALD</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PFL</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>PFL, FDH</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>ACL</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>ACS</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PK, PTA(^a)</td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>PK, PTA(^b)</td>
<td>2/5</td>
<td>0</td>
</tr>
</tbody>
</table>

\(^a\) Xylose-5-phosphate was obtained from glucose-6-phosphate through the pentose phosphate pathway. Glyceraldehyde-3-phosphate produced by PK was converted to acetyl-CoA through glycolysis and the native yeast pathway for acetyl-CoA synthesis.

\(^b\) Xylose-5-phosphate was obtained from fructose-6-phosphate and glyceraldehyde-3-phosphate through the nonoxidative part of the pentose phosphate pathway.

**TABLE 5** Saccharomyces cerevisiae strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Relevant genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>CEN.PK113-7D</td>
<td>MATA MAL2-8° SUC2</td>
<td>P. Kotter</td>
</tr>
<tr>
<td>CEN.PK102-12A</td>
<td>MATA MAL2-8° SUC2 ura3-52 leu2-3,112 his3-Δ1</td>
<td>P. Kotter</td>
</tr>
<tr>
<td>IMK427</td>
<td>CEN.PK102-12A ascl-2::loxP-Sphis5-loxP</td>
<td>18</td>
</tr>
<tr>
<td>IMZ466</td>
<td>IMK427 pUDE333</td>
<td>This study</td>
</tr>
<tr>
<td>IMY102</td>
<td>IMK427 pUDC140</td>
<td>This study</td>
</tr>
<tr>
<td>IMY104</td>
<td>IMY102 ascl-2::loxP-KIUE2-lopX</td>
<td>This study</td>
</tr>
<tr>
<td>IMY109</td>
<td>IMK427 pUDC143</td>
<td>This study</td>
</tr>
<tr>
<td>IM0176</td>
<td>IMATB ura3-52 pdc1(Δ−6,−2)::loxP pdc5Δ(−6,−2)::loxP pdc6Δ(−6,−2)::loxP MTH1-ΔT</td>
<td>53</td>
</tr>
<tr>
<td>IMX216</td>
<td>IM0176 pda1::loxP-KanMX4-lopX</td>
<td>This study</td>
</tr>
<tr>
<td>IMQ011</td>
<td>IMX216 pUDC140</td>
<td>This study</td>
</tr>
<tr>
<td>IMU064</td>
<td>IMX216 pUDE333</td>
<td>This study</td>
</tr>
</tbody>
</table>

\(\text{IM}+\) strains used in this study (Table 5) share the CEN.PK genetic background (45, 46). Strain construction. \textit{S. cerevisiae} strains were transformed according to the methods described by Gietz et al. (51). Knockout cassettes with \textit{KanMX}, \textit{Sphis}5, and \textit{KIUE2} were obtained by PCR using the tailored primers listed in Table S3 in the supplemental material, with the templates pUG6, pUG27, and pUG73 (52), respectively, to generate genetic markers flanked by sequence with homology to chromosomal loci for targeting by homologous integration. Mutants were selected on solid medium (2% [wt/vol] agar) with 200 mg liter\(^{-1}\) G418 or on dropout medium (Sigma-
Aldrich) or synthetic medium from which the appropriate auxotrophic requirements had been omitted. The IMK427 strain was constructed as described previously (18). Assembly of plasmids pUDE333, pUDC140, and pUDC143 in IMK427 resulted in strains IMZ466, IMY102, and IMY109, respectively. In one of the resulting strains, IMY102, ACS1 was subsequently deleted, yielding IMY104. The IMX216 strain was obtained by deletion of PDA1 in the IMI076 strain (53). Transformation of IMX216 with plasmids pUDC140 and pUDE333 resulted in strains IMQ011 and IMU064, respectively. In all cases, gene deletion and/or plasmid presence were confirmed by PCR using the diagnostic primers listed in Table S3.

Molecular biology techniques. PCR amplification with Phusion Hot Start II high-fidelity polymerase (Thermo Scientific, Waltham, MA) was performed according to the manufacturer’s manual using high-performance liquid chromatography (HPLC)- or PAGE-purified, custom-synthesized oligonucleotide primers (Sigma-Aldrich). Diagnostic PCR was done with DreamTaq (Thermo Scientific) and desalted primers (Sigma-Aldrich). DNA fragments obtained by PCR were loaded on gels containing 1% or 2% (wt/vol) agarose (Thermo Scientific) and 1× Tris-acetate-EDTA buffer (Thermo Scientific), excised, and purified (Zymoclean, D2004; Zymo Research, Irvine, CA). Alternatively, fragments were purified using the GenElute PCR Cleanup kit (Sigma-Aldrich). Plasmids were isolated from E. coli with the Sigma GenElute Plasmid kit (Sigma-Aldrich) according to the manufacturer’s instructions. Yeast plasmids were isolated according to the methods described in reference 50. Yeast genomic DNA was isolated using a Yeast genomic DNA kit (Zymo Research). E. coli DH5α (1828-012; Invitrogen) was transformed chemically (T3001; Zymo Research) or by electroporation. Chemical transformation was done according to the supplier’s instructions. Electroporation was done in a 2-mm cuvette (165-2086; Bio-Rad, Hercules, CA) by using a Gene PulserXcell electroporation system (Bio-Rad), following the manufacturer’s protocol.

Media and cultivation. Shake-flask cultures were grown at 30°C in 500-ml flasks containing 100 ml synthetic medium (47) with 20 g liter−1 glucose in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ) set at 200 rpm. When required, media were supplemented with lipic acid at the concentration of 50 mg ml−1. Optical density at 600 nm was measured at regular time intervals with a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom). Chemostat cultivations were carried out at 30°C in 2-liter laboratory bioreactors (Applikon, Schiedam, The Netherlands) with working volumes of 1 liter. Chemostat cultivation was preceded by a batch phase under the same conditions. When a rapid decrease in CO2 production indicated glucose depletion in the batch cultures, continuous cultivation at a dilution rate of 0.10 h−1 was initiated. Synthetic medium (47) supplemented with 7.5 g liter−1 glucose was used. Lipoic acid solution in ethanol was prepared separately and added to the medium to a final concentration of 500 ng ml−1. Antifoam Pluronic PE 6000 (BASF, Ludwigshafen, Germany) was added to the media before sterilization to a final concentration of 0.15 g liter−1. Culture pH was maintained at 5.0 by automatic addition of 2 M KOH. Aerobic bioreactors were sparged with 500 ml min−1 air and stirred at 800 rpm to ensure fully aerobic conditions.

Analytical methods. Chemostat cultures were assumed to be in steady state when, after at least 5 volume changes, the carbon dioxide production rates changed by less than 2% over 2 volume changes. Steady-state samples were taken between 12 and 17 volume changes after inoculation. Dry weight measurements were performed as described previously (54). Off-gas was first cooled in a condenser (2°C) and dried with a Perma Pure dryer (Perma Pure LLC, Toms River, NJ). CO2 and O2 concentrations in the off-gas were measured with an NGA 2000 Rosemount gas analyzer (Rosemount Analytical Inc., Orrville, OH). Ethanol concentrations were corrected for evaporation, as described by Guadalupe Medina et al. (55). Samples for residual glucose and ethanol determinations were taken with the stainless steel bead method for rapid quenching of metabolites (56). HPLC analyses of the supernatant and of residual nutrients were performed as described previously (54).

Enzymatic determination of metabolites. Ethanol was measured using an ethanol assay kit (Sigma-Aldrich) according to the manufacturer’s instructions. Extracellular lycorol was measured using the lycorl enzymatic determination kit (10148270035; R-Biopharm AG, Darmstadt, Germany). Measurements were done according to the manufacturer’s instructions, except that the volumes for the assays were proportionally downscaled (final volume, 0.3 ml). Absorbance was measured using 96-well plates (at least three replicates per sample) on a GENios Pro apparatus (Tecan, Giessen, Netherlands).

PDH complex and acetyl-CoA synthetase activity assay. Biomass for enzyme analysis (corresponding to ca. 125 mg dry weight) was harvested from exponentially growing shake flask cultures, washed twice (5 min, 4,600 × g at 4°C) in 10 mM potassium phosphate buffer (pH 7.5) with 1 mM EDTA and stored at −20°C. Prior to preparation of cell extracts, samples were thawed on ice, washed, and resuspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM MgCl2 and 1 mM di-thiothreitol (prepared fresh). Cell extracts were prepared by a Fast Prep method (4 bursts of 20 s with 30-s intervals at 0°C) on a Fast Prep FP120 system (Thermo Scientific Corporation, Waltham, MA). After removal of cells and debris by centrifugation (4°C, 20 min at 47,000 × g), the supernatant was used for enzyme assays. Protein concentrations in cell extracts were measured with the Lowry method (57), and bovine serum albumin (BSA; essentially fatty acid free) from Sigma-Aldrich was used as a standard. Pyruvate decarboxylases are known to interfere with measurements of the enzymatic activity of the pyruvate dehydrogenase complex (58); therefore, the wild-type CEN.PK113-7D (Pdc+), and Pdc− strains were used for optimization of the enzymatic assay. In addition, to specifically measure the activity of the introduced E. faecalis PDH, the PDA1 gene coding for the E1α subunit of the native PDH complex of yeast was deleted, thereby eliminating the activity of the native PDH complex (59). Pyruvate dehydrogenase (EC 1.2.1.51) activity was measured at 30°C on a
Hitachi model 100-60 spectrophotometer (Systron, Norderstedt, Germany) by monitoring the reduction of NAD\(^+\) at 340 nm in a 1:m reaction mixture containing 15 mM pyrazole, 100 mM phosphate buffer (pH 8.0), 1 mM MgCl\(_2\), 0.2 mM thiamine pyrophosphate, 2.5 mM NAD\(^+\), 5 mM pyruvate, 2 mM L-cysteine-HCl (prepared fresh), 0.035% (vol/vol) Triton X-100 (prepared fresh), and 20 to 100 μl of cell extract. The reaction was started by addition of 0.15 mM coenzyme A. The acetyl-CoA synthetase activity (EC 6.2.1.1) was measured as described previously (28). Specific activities are expressed as micromole of substrate converted per minute per milligram of protein. Reaction rates were proportional to the amount of cell extract added.

Separation of mitochondrial and cytosolic fractions. The separation of mitochondrial and cytosolic fractions was performed as described previously by Luttik et al. (29) with minor modifications. Zymolysis of Arthrobacter luteus (20,000 U g\(^{-1}\); AMS Biotechnology Ltd., Abingdon, United Kingdom) was used. The CEN.PK113-7D and IMY104 biomass was harvested from glucose-limited, aerobic chemostat cultures supplemented with 0.05% (vol/vol) Triton X-100, 25 mM potassium phosphate buffer [pH 7.5], 1 mM EDTA, and 100 mM potassium phosphate buffer (pH 7.0), containing 0.01% sodium azide, 5% glycerol, 0.1 mM ribosylthymine phosphate, and 0.1 mM di-thiothreitol (60). The protein sample was applied to a HiPrep 16/60 Sephacyr S-300 HR chromatographic column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The elution was performed with the same potassium buffer (pH 7.0), at a flow rate of 0.5 ml min\(^{-1}\). Elution of proteins was followed spectrophotometrically at 280 nm. Fractions (2 ml each) were collected and used to measure PDH activity as described above. The protein contents of the fractions were determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories Inc., Hercules, CA) according to the supplier’s manual.

Purification of the PDH complex on chromatographic columns. The separation of mitochondrial and cytosolic fractions of the IMY104 strain was performed as described above. The cytosolic fraction was subsequently centrifuged (4°C, 10 min at 47,000 x g). The obtained supernatant was transferred to an Amicon Ultra-15 centrifugal unit (100-kDa cutoff; EMD Millipore Corporation, Billerica, MA) for protein concentration and buffer replacement. Sorbitol-containing buffer (0.65 M sorbitol, 25 mM potassium phosphate buffer [pH 7.5], 1 mM EDTA, and 1 mM MgCl\(_2\)), used in the process of cellular fractionation, was replaced with 100 mM potassium phosphate buffer (pH 7.0), containing 0.01% sodium azide, 5% glycerol, 0.1 mM ribosylthymine phosphate, and 0.1 mM di-thiothreitol (60). The protein sample was applied to a HiPrep 16/60 Sephacyr S-300 HR chromatographic column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom). The elution was performed with the same potassium buffer (pH 7.0), at a flow rate of 0.5 ml min\(^{-1}\). Elution of proteins was followed spectrophotometrically at 280 nm. Fractions (2 ml each) were collected and used to measure PDH activity as described above. The protein contents of the fractions were determined using the Quick Start Bradford protein assay (Bio-Rad Laboratories Inc.) according to the supplier’s manual.

Proteomic analysis. The protein concentrations in samples for proteomic analysis were determined by using the Quick Start Bradford protein assay (Bio-Rad Laboratories Inc.) according to the supplier’s manual. The protein concentration in the cytosolic fraction aliquots, obtained as described above, was normalized to 1 mg ml\(^{-1}\) with demineralized water. The pellet containing mitochondria was resuspended in methanol to a final protein concentration of 1 mg ml\(^{-1}\). The protein concentration in fractions obtained from the PDH purification by gel filtration was below 1 mg ml\(^{-1}\), and therefore normalization to 1 mg ml\(^{-1}\) was not required. However, in the cases of column fractions 14 and 16, freeze-drying (61) followed by resuspension in demineralized water was applied in order to concentrate the sample. BSA was added to all samples to a final concentration of 1% (wt/wt) of total protein. Trichloroacetic acid at 20% (wt/vol) was added in a 1:1 volume ratio. The protein precipitation was carried out on ice for 2 h. After incubation, the samples were centrifuged (4°C, 10 min at 16,000 x g). The pellets were washed with acetone (−20°C) and stored at −20°C. Chloroform was added (1:1 volumetric ratio) to the samples containing mitochondria resuspended in methanol. Samples were vortexed for 1 min. Subsequently, 20% (wt/vol) trichloroacetic acid was added in a 1:1 volume ratio, and samples were vortexed for 1 min. The chloroform–20% (wt/vol) trichloroacetic acid procedure was repeated. Samples were agitated for 60 min at room temperature on a vortexer (DVX-2500; Multiple Tube Vortexer, VWR International, Amsterdam, The Netherlands). Finally, samples were centrifuged (4°C, 10 min at 16,000 x g) and the pellets were washed with acetone (−20°C) and stored at −20°C. The HPLC-tandem mass spectrometry (MS/MS)-based absolute protein expression profiling analysis was performed as described previously (62).

RNA-seq transcriptome analysis. Sampling for transcriptome analysis from chemostat cultures and total RNA extraction was performed as described previously (63). Sequencing was performed using an Illumina HiSeq 2500 apparatus and carried out by Baseclear (Leiden, The Netherlands). Data sets of 100–bp paired end reads of at least 1 Gb were generated. The genome sequence of CEN.PK113-7D (46) was used as the reference genome for all analysis. The data were aligned to the reference by using the Burrow-Wheeler alignment tool BWA (64). Gene expression levels were estimated using FPKM values by using the Cufflinks software (65). To identify differential gene expression between strains CEN.PK113-7D and IMY104, RNA-seq data comparison was performed and statistically assessed using Cuffdiff (65). Overrepresentation of functional categories in sets of differentially expressed genes was analyzed according to methods described previously (66).

Microarray data accession number. RNA-seq data generated in this study were submitted to the Genome Expression Omnibus database and assigned accession number GSE59914.

SUPPLEMENTAL MATERIAL
Supplemental material for this article may be found at http://mbio.asm.org/lookup/suppl/doi:10.1128/mBio.01696-14/-/DCSupplemental.

Table S1, DOCX file, 0.02 MB.
Table S2, DOCX file, 0.02 MB.
Table S3, DOCX file, 0.02 MB.

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REFERENCES