CrossMark
← click for updates

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

Barbara U. Kozak,[a] Harmen M. van Rossum,[a] Marijke A. H. Luttik,[a] Michiel Akeroyd,[c] Kirsten R. Benjamin,[b] Liang Wu,[c] Simon de Vries,[a] Jean-Marc Daran,[a] Jack T. Pronk,[a] Antonius J. A. van Maris[a]

Department of Biotechnology, Delft University of Technology, Delft, The Netherlands[a]; Amyris Inc., Emeryville, California, USA[b]; DSM Biotechnology Center, Delft, The Netherlands[c]

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 Acs⁺ 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.

Pyruvate dehydrogenase (PDH), a ubiquitous protein complex found in all domains of life, catalyzes the oxidative decarboxylation of pyruvate to acetyl coenzyme A (acetyl-CoA), a reaction that forms the interface between glycolysis and the tricarboxylic acid cycle (TCA). PDH consists of three catalytic subunits: pyruvate dehydrogenase (E1), dihydrolipoyl transacetylase (E2), and dihydrolipoyl dehydrogenase (E3), which together form multimeric complexes whose size, in many organisms, exceeds that of a ribosome (1). In eukaryotes and some bacteria, E1 consists of two proteins (E1α and E1β) (1). PDH requires four different cofactors. Thiamine pyrophosphate is bound to E1 and participates in pyruvate decarboxylation; lipoate is covalently attached to a con-

served lysine residue of E2 and mediates translocation of intermediates between the active sites of E1, E2, and E3, while FAD and NAD⁺ are required for reoxidation of dihydrolipoate by E3. A complex architecture, involving up to 60 copies per subunit in one PDH complex, enables efficient coordination of the E1, E2, and E3 activities and is, in some organisms, the result of self-assembly (2, 3). In other organisms, assembly requires additional proteins (4).

The product of the PDH reaction, acetyl-CoA, is a crucial precursor for biosynthesis of a wide variety of biomolecules. These include many industrially relevant compounds, including *n*-butanol, lipids, isoprenoids, flavonoids, and 3-hydroxypropionic acid (5–10). Expression of heterologous and

Kozak et al.

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 metabolic 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 C_{16} 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 (A-ALD) 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 lipoate-protein ligase LplJ of *Bacillus subtilis* (24). Codon-optimized genes encoding the E1α, E1β, E2, and E3 subunits of *Enterococcus faecalis* PDH encoded by *pdhA*, *pdhB*, *aceF*, and *lpd*, 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* transcription is repressed and the Acs1 protein is inactivated; thus, *acs2Δ* mutants cannot grow on glucose plates (27, 28). Since Acs1 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*, *lpd*, *lplA*, and *lplA2*) and IMY102 (*acs2Δ* pUDC140; centromeric vector with expression cassettes for *pdhA*, *pdhB*, *aceF*, *lpd*, *lplA*, and *lplA2*). Additionally, strain IMY109 (*acs2Δ* pUDC143; centromeric vector with expression cassettes for *pdhA*, *pdhB*, *aceF*, and *lpd* 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.
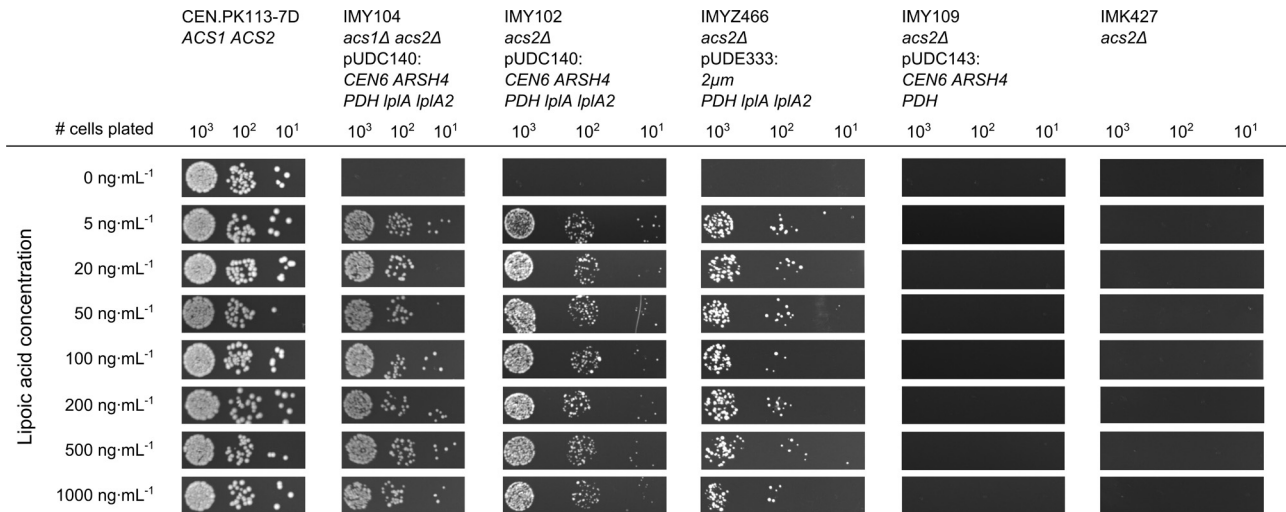
FIG 1 Growth of *S. cerevisiae* strains CEN.PK113-7D (*ACS1 ACS2*), IMK427 (*acs2Δ*), IMZ466 (*acs2Δ* pUDE333; 2μ *pdhA pdhB aceF lpd lplA lplA2*), IMY102 (*acs2Δ* pUDC140; *CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*), IMY104 (*acs1Δ acs2Δ* pUDC140; *CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*), and IMY109 (*acs2Δ* pUDC143; *CEN 6ARS4 pdhA pdhB aceF lpd*) on synthetic medium agar plates with 2% (vol/vol) ethanol, 20 g liter^{-1} glucose, or 20 g liter^{-1} glucose supplemented with lipoic acid. When needed, uracil and leucine were added to cover the auxotrophic requirements. Plates with strains CEN.PK113-7D, IMY102, and IMY104 were incubated aerobically for 48 h, while plates with strains IMK427, IMZ466, and IMY109 were incubated aerobically for 96 h. PDH in strain descriptions represents the following set of genes: *pdhA pdhB aceF lpd*.

However, when lipoic acid was included in the growth medium, strains IMZ466 and IMY102, which expressed the *E. faecalis* PDH subunit genes and lipoylation genes, did grow on glucose. Under the same conditions, neither IMK427 (*acs2Δ*) nor IMY109 (*acs2Δ*, 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 *Enterococus faecalis*[a]

Strain	Relevant genotype	Enzyme sp act (nmol/min/mg of protein)	
		PDH	ACS
CEN.PK113-7D	*ACS1 ACS2 PDC1 PDC5 PDC6 PDA1*	12 ± 2	116 ± 18
IMY104	*acs1Δ acs2Δ pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*	53 ± 2	BDL
IMX216	*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1-ΔT*	BDL	666 ± 13
IMU064	*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1-ΔT pUDE333; 2μ pdhA pdhB aceF lpd lplA lplA2*	18 ± 1	ND
IMQ011	*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1-ΔT pUDC140; CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*	30 ± 1	ND

[a] 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 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.
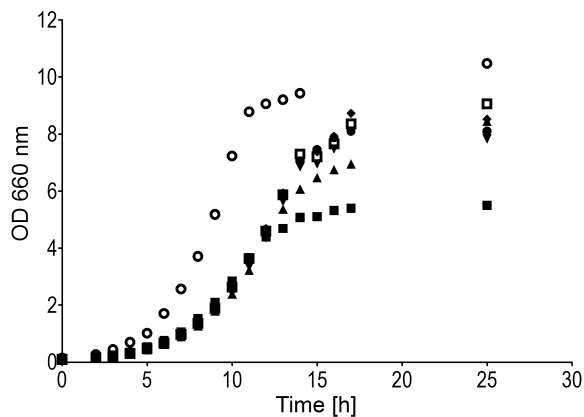
FIG 2 Growth curves and final optical densities of cultures of *S. cerevisiae* strains IMY104 (*acs1Δ acs2Δ* pUDC140; *CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*) and CEN.PK113-7D (*ACS1 ACS2*) grown on synthetic medium with 20 g liter^{-1} glucose, supplemented with different concentrations of lipoic acid. Strain CEN.PK113-7D had a specific growth rate of 0.42 h^{-1}. The growth rate of the IMY104 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; 2μ *pdhA pdhB aceF lpd lplA lplA2*) and IMQ011 (*pdc1Δ pdc5Δ pdc6Δ pda1Δ MTH1-ΔT* pUDC140; *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.
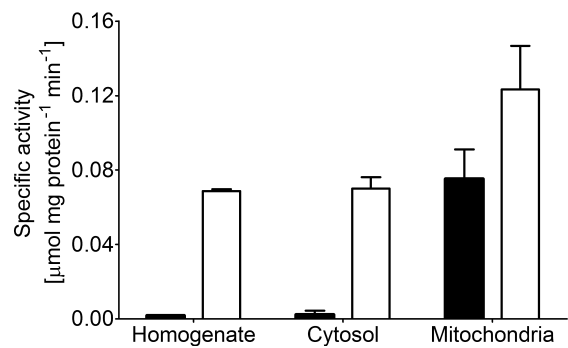

FIG 3 Specific activities of the pyruvate dehydrogenase complex measured in the total homogenate (cytosolic and mitochondrial fractions not separated) and in cytosolic or mitochondrial fractions of *S. cerevisiae* strains IMY104 (*acs1Δ acs2Δ* pUDC140; *CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*) and CEN.PK113-7D (*ACS1 ACS2*). White bars, IMY104; black bars, CEN.PK113-7D. Averages and standard deviations were obtained from two replicate experiments.

To check 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*) were separated by subcellular fractionation (29). The same procedure was applied to the reference strain CEN.PK113-7D (*ACS1 ACS2*). In the latter strain, PDH activity was predominantly found in the mitochondrial fraction (Fig. 3). Conversely, in strain IMY104, which contained both the native mitochondrial PDH complex as well as the introduced complex from *E. faecalis*, PDH activity was found in both the cytosolic and mitochondrial fractions. However, while the specific activities of PDH in the mitochondrial fraction of IMY104 and CEN.PK113-7D were not significantly different, the specific activity of PDH in the cytosolic fraction of IMY104 was 32-fold higher than that of the wild-type strain (Fig. 3). This suggests that the PDH of *E. faecalis* is indeed expressed in the cytosol of *S. cerevisiae*.

Mass spectrometry-based proteome analysis confirmed the presence of all four subunits of PDH of *E. faecalis*, as well as the two lipoate-protein ligases, in the cytosolic fraction of strain IMY104. Although all six proteins were also detected in the mitochondrial fraction, their relative levels were much higher in the cytosolic fraction than in the mitochondrial fraction (data not shown). The presence of those proteins in the mitochondrial fraction may be caused by contamination of that fraction during the separation procedure. However, partial targeting of those proteins to the mitochondrion cannot be excluded. The three subunits of the native yeast pyruvate dehydrogenase complex Pdb1 (pyruvate dehydrogenase beta-subunit [E1β]), Lat1 (dihydrolipoamide acetyltransferase [E2]), and Pdx1 (pyruvate dehydrogenase complex protein X), were exclusively detected in the mitochondrial fraction. Together, these results confirm that the PDH activity found in the cytosolic fraction originates from the heterologously expressed *E. faecalis* PDH.

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
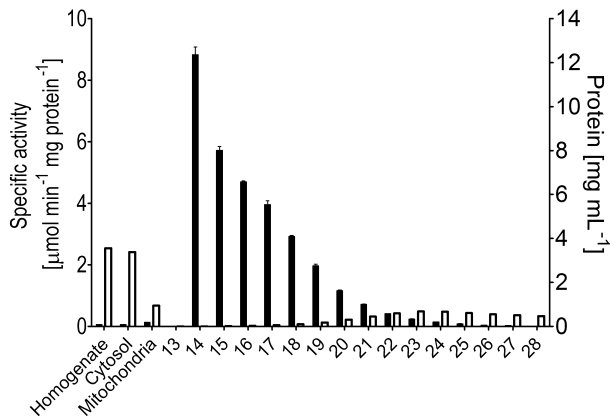
FIG 4 Specific activities of pyruvate dehydrogenase complex (black bars), as well as protein levels (white bars) measured in the total homogenate (cytosolic and mitochondrial fractions not separated), in cytosolic fractions, and in mitochondrial fraction of *S. cerevisiae* IMY104 (*acs1Δ acs2Δ* pUDC140; *CEN6 ARS4 pdhA pdhB aceF lpd lplA lplA2*), as well as in column fractions 13 to 28, obtained during purification of the PDH complex present in the cytosol on the chromatographic column. In cases of fractions obtained from the chromatographic column, the PDH activity was observed in fractions 14 to 27. Averages and standard deviations were obtained from two measurements performed for the same sample. This single experiment was qualitatively representative of duplicate experiments.

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, which 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 $\pm$ 0.19:1 $\pm$ 0.10:1.26 $\pm$ 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^{-1}. 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

TABLE 3 Physiological characterization of the *S. cerevisiae* reference strain CEN.PK113-7D and strain IMY104[a]

Parameter	Mean $\pm$ SD value for strain	
	CEN.PK113-7D	IMY104
Dilution rate (h^{-1})	0.099 $\pm$ 0.002	0.100 $\pm$ 0.001
Biomass yield (g of biomass/g of glucose)	0.496 $\pm$ 0.007	0.482 $\pm$ 0.004
$q_{glucose}$ (mmol/g of biomass/h)	-1.10 ± 0.01	-1.15 ± 0.02
$q_{ethanol}$ (mmol/g of biomass/h)	ND	ND
q_{CO2} (mmol/g of biomass/h)	2.81 $\pm$ 0.03	3.00 $\pm$ 0.07
q_{oxygen} (mmol/g of biomass/h)	-2.71 ± 0.05	-2.82 ± 0.08
$q_{pyruvate}$ (mmol/g of biomass/h)	ND	ND
$q_{glycerol}$ (mmol/g of biomass/h)	0.006 $\pm$ 0.001	0.007 $\pm$ 0.000
$q_{acetate}$ (mmol/g of biomass/h)	0.014 $\pm$ 0.002	0.018 $\pm$ 0.000
Residual glucose (g/liter)	0.021 $\pm$ 0.001	0.024 $\pm$ 0.001
Carbon recovery (%)	103 $\pm$ 1	102 $\pm$ 1

[a] Strains were grown in aerobic, glucose-limited chemostat cultures supplemented with 500 ng/ml lipoic acid, at a dilution rate of 0.10 h^{-1}. Means and standard deviations were obtained from three independent cultures for each strain. ND, not detected.

TABLE 2 Purification of the PDH complex of *E. faecalis* expressed in *S. cerevisiae*[a]

Sample	Purification step	Sp act (μmol/min/mg of protein)	Purification factor
Homogenate		0.067 $\pm$ 0.003	1
Cytosol C1	Centrifugation	0.062 $\pm$ 0.001	0.9
Cytosol C2	Centrifugation	0.071 $\pm$ 0.003	1.1
Cytosol C3	Concentration on 100-kDa cutoff filter	0.083 $\pm$ 0.003	1.2
Column fraction 14	Column separation	8.83 $\pm$ 0.25	131

[a] 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 (*acs1Δ acs2Δ* p*UDC140*; *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 ≥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 *lpd*—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 medim, which was presumably used by the lipoate ligases for lipoylation of the cytosolically 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, which makes it difficult to interpret their results (40).

PDH of *E. faecalis* is 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.

Replacement of the native route to cytosolic acetyl-CoA with a multisubunit bacterial PDH generated a strain with physiological properties nearly indistinguishable from those of the original parental strain. Experiments in an *acs1Δ acs2Δ S. cerevisiae* genetic background showed that cytosolic expression of *E. faecalis* PDH and lipoylation genes, combined with the addition of lipoate to the growth medium, could functionally replace the *S. cerevisiae* PDH bypass as the sole pathway for synthesis of cytosolic acetyl-CoA in glucose-grown cultures. The thus-engineered strain showed near-wild-type growth rates and biomass yield on glucose in aerobic cultures. In many PDH complexes, the activity of the E3 subunit is strongly inhibited at high NADH/NAD$^+$ ratios, 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 inhibition than PDH complexes from other microorganisms (19). Functionality under anaerobic conditions may be an important asset for use of the *E. faecalis* PDH complex in yeast metabolic engineering studies aimed at anaerobic 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 the glyceraldehyde-3-phosphate formed 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 ATP-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 positive net ATP yield for synthesis of acetyl-CoA from glucose (Table 4). However, replacement of the native yeast pathway for cytosolic acetyl-CoA synthesis by either of these pathways led to a decreased biomass yield compared to the Acs$^+$ reference strain, probably as a result of the accumulation of intermediates or by-

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

Pathway	Molar ratio		Reference
	ATP/acetyl-CoA	NADH/acetyl-CoA	
PDH	1	2	This study
A-ALD	1	2	18
PFL	1	1	18
PFL, FDH	1	2	18
ACL	0	2	17
ACS	−1	2	8
PK, PTA[a]	−1/2	2	44
PK, PTA[b]	−2/5	0	44

[a] Xylulose-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] Xylulose-5-phosphate was obtained from fructose-6-phosphate and glyceraldehyde-3-phosphate through the nonoxidative part of the pentose phosphate pathway. Glyceraldehyde-3-phosphate produced by PK was (partially) recycled to produce fructose-6-phosphate.

products (18). The absence of a clear phenotype of PDH-dependent *S. cerevisiae* indicates that cytosolic expression of *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 *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 *E. faecalis* PDH should not be cost prohibitive. Assuming 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 *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 ng ml^{-1}. Carbon sources were either 20 g liter^{-1} glucose or 2% (vol/vol) ethanol. Frozen stocks of *S. cerevisiae* and *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°C.

Plasmid construction. Protein sequences of *Enterococcus faecalis* PDH subunits, component E1α (*pdhA*; accession number YP_005708198.1), component E1β (*pdhB*; YP_005708199.1), component E2 (dihydrolipoamide acetyltransferase; *aceF*; YP_005708200.1), and component E3 (dihydrolipoyl dehydrogenase; *lpd*; YP_005708201.1), as well as the genes responsible for attachment of lipoic acid into the E2 subunit of PDH, lipoate-protein ligase *lplA* (YP_005707438.1) and lipoate-protein ligase *lplA2* (YP_005709162.1), were used to obtain codon-optimized gene sequences. The codon optimization was performed according to the algorithm described previously (49). The sequences of promoters and/or terminators of the genes *ADH1*, *CYC1*, *PGI1*, *PGK1*, *PMA1*, *PYK1*, *TDH3*, *TEF1*, and *TPI1* were derived from the CEN.PK113-7D genome sequence (46). Custom-synthesized coding sequences flanked by the appropriate promoter and terminator, sequences enabling homologous recombination and specific restriction sites (see Table S1 in the supplemental material), were provided by GeneArt GmbH (Regensburg, Germany) cloned in the pUC57 vector (Table 6). The sequences of *S. cerevisiae* replicons 2μ and CEN6 ARS4 (originating from plasmids pRS426-TEF1 and pRS316, respectively), sequences of the orotidine-5′-phosphate (OMP) decarboxylase *URA3* gene with its promoter and terminator, and the *E. coli* replication cassette pMB1 with ampicillin resistance gene *AmpR* (taken from the pRS416 plasmid), all flanked with sequences enabling homologous recombination and specific restriction sites, were provided by GeneArt GmbH (Regensburg, Germany) cloned in the pUC57 vector (Table 6). Sequences of all fragments are given in Table S2 in the supplemental material. Plasmids were transformed into *E. coli* for storage and amplification. The expression cassettes were PCR amplified with appropriate combinations of primers (see Table S3 in the supplemental material). Plasmid assembly via *in vivo* homologous recombination (50) in *S. cerevisiae* strains IMK427 and IMX216 resulted in plasmids pUDC140, pUDC143, and pUDE333 (Table 6). The sequences of the introduced genes of the pyruvate dehydrogenase complex as well as the genes coding for lipoyl-protein ligases *lplA* and *lplA2* were confirmed by RNA sequencing (see "RNA-seq transcriptome analysis" below).

Strain construction. *S. cerevisiae* strains were transformed according to the methods described by Gietz et al. (51). Knockout cassettes with *KanMX*, *Sphis5*, and *KlLEU2* were obtained by PCR using the tailed 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-

TABLE 5 *Saccharomyces cerevisiae* strains used in this study

Name	Relevant genotype	Origin
CEN.PK113-7D	*MAT**a** MAL2-8^C SUC2*	P. Kötter
CEN.PK102-12A	*MAT**a** MAL2-8^C SUC2 ura3-52 leu2-3,112 his3-Δ1*	P. Kötter
IMK427	CEN.PK102-12A *acs2::loxP-Sphis5-loxP*	18
IMZ466	IMK427 pUDE333	This study
IMY102	IMK427 pUDC140	This study
IMY104	IMY102 *acs1::loxP-KlLEU2-loxP*	This study
IMY109	IMK427 pUDC143	
IMI076	*MAT**a** ura3-52 pdc1Δ(−6, −2)::loxP pdc5Δ(−6, −2)::loxP pdc6Δ(−6, −2)::loxP MTH1-ΔT*	53
IMX216	IMI076 *pda1::loxP-KanMX4-loxP*	This study
IMQ011	IMX216 pUDC140	This study
IMU064	IMX216 pUDE333	This study

TABLE 6 Plasmids used in this study

Name	Characteristics	Origin
pUC57	Delivery vector	GeneArt, Germany
pUG6	Template for loxP-KanMX-loxP cassette	52
pUG27	Template for loxP-Sphis5-loxP cassette	52
pUG73	Template for loxP-KlLEU2-loxP cassette	52
pRS426-TEF1	Sequence template for 2μ	67
pRS316	Sequence template for CEN6 ARS4	68
pRS416	Sequence template for URA3 (including promoter and terminator)	68
pUDC140	URA3 CEN6 ARS4 pTPI1-pdhA-tTEF1 pTDH3-pdhB-tCYC1 pADH1-aceF-tPGI1 pTEF1-lpd-tADH1 pPGK1-lplA-tPMA1 pPGI1-lplA2-tPYK1 pMB1-AmpR	This study
pUDC143	URA3 CEN6 ARS4 pTPI1-pdhA-tTEF1 pTDH3-pdhB-tCYC1 pADH1-aceF-tPGI1 pTEF1-lpd-tADH1 pMB1-AmpR	This study
pUDE333	URA3 2μ pTPI1-pdhA-tTEF1 pTDH3-pdhB-tCYC1 pADH1-aceF-tPGI1 pTEF1-lpd-tADH1 pPGK1-lplA-tPMA1 pPGI1-lplA2-tPYK1 pMB1-AmpR	This study

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 supplier's manual. Yeast plasmids were isolated according to the methods described in reference 50. Yeast genomic DNA was isolated using a YeaStar genomic DNA kit (Zymo Research). *E. coli* DH5α (18258-012; 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 lipoic acid at the concentration of 50 ng ml^{-1}. Optical density at 660 nm was measured at regular time intervals with a Libra S11 spectrophotometer (Biochrom, Cambrige, 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 CO_2 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 6100 (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). CO_2 and O_2 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 glycerol was measured using the glycerol 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 $MgCl_2$ and 1 mM dithiothreitol (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 (Sysmex, Norderstedt, Germany) by monitoring the reduction of NAD$^+$ at 340 nm in a 1-ml 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), 0.05% (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. Zymolyase from *Arthobacter 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 500 ng ml^{-1} of lipoic acid. PDH activity was measured in the homogenate as well as in the obtained cytosolic and mitochondrial fractions. 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 × *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 fraction, 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 dithiothreitol (60). The protein sample was applied to a HiPrep 16/60 Sephracyl S-500 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 × *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 × *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 GSE59814.

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.

ACKNOWLEDGMENTS

REFERENCES

1. **Patel MS, Roche TE.** 1990. Molecular biology and biochemistry of pyruvate dehydrogenase complexes. FASEB J. **4:**3224–3233.
2. **Domingo GJ, Chauhan HJ, Lessard IA, Fuller C, Perham RN.** 1999. Self-assembly and catalytic activity of the pyruvate dehydrogenase multienzyme complex from *Bacillus stearothermophilus*. Eur. J. Biochem. **266:** 1136–1146. http://dx.doi.org/10.1046/j.1432-1327.1999.00966.x.
3. **Perham RN, Hooper EA.** 1977. Polypeptide chain stoichiometry in the self-assembly of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. FEBS Lett. **73:**137–140. http://dx.doi.org/10.1016/0014-5793(77)80965-4.
4. **Witzmann S, Bisswanger H.** 1998. The pyruvate dehydrogenase complex from thermophilic organisms: thermal stability and re-association from the enzyme components. Biochim. Biophys. Acta **1385:**341–352. http://dx.doi.org/10.1016/S0167-4838(98)00078-8.
5. **Chen Y, Bao J, Kim IK, Siewers V, Nielsen J.** 2014. Coupled incremental precursor and co-factor supply impr 3-hydroxypropionic acid production in *Saccharomyces cerevisiae*. Metab. Eng. **22:**104–109. http://dx.doi.org/10.1016/j.ymben.2014.01.005.
6. **Dyer JM, Chapital DC, Kuan JW, Mullen RT, Pepperman AB.** 2002. Metabolic engineering of *Saccharomyces cerevisiae* for production of novel lipid compounds. Appl. Microbiol. Biotechnol. **59:**224–230. http://dx.doi.org/10.1007/s00253-002-0997-5.
7. **Koopman F, Beekwilder J, Crimi B, van Houwelingen A, Hall RD, Bosch D, van Maris AJ, Pronk JT, Daran JM.** 2012. *De novo* production of the flavonoid naringenin in engineered *Saccharomyces cerevisiae*. Microb. Cell Fact. **11:**155. http://dx.doi.org/10.1186/1475-2859-11-155.
8. **Shiba Y, Paradise EM, Kirby J, Ro DK, Keasling JD.** 2007. Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for

high-level production of isoprenoids. Metab. Eng. **9**:160–168. http://dx.doi.org/10.1016/j.ymben.2006.10.005.

9. **Steen EJ, Chan R, Prasad N, Myers S, Petzold CJ, Redding A, Ouellet M, Keasling JD.** 2008. Metabolic engineering of *Saccharomyces cerevisiae* for the production of *n*-butanol. Microb. Cell Fact. **7**:36. http://dx.doi.org/10.1186/1475-2859-7-36.

10. **Veen M, Lang C.** 2004. Production of lipid compounds in the yeast *Saccharomyces cerevisiae*. Appl. Microbiol. Biotechnol. **63**:635–646. http://dx.doi.org/10.1007/s00253-003-1456-7.

11. **Hong KK, Nielsen J.** 2012. Metabolic engineering of *Saccharomyces cerevisiae*: a key cell factory platform for future biorefineries. Cell. Mol. Life Sci. **69**:2671–2690. http://dx.doi.org/10.1007/s00018-012-0945-1.

12. **Pronk JT, Yde Steensma H, van Dijken JP.** 1996. Pyruvate metabolism in *Saccharomyces cerevisiae*. Yeast **12**:1607–1633. http://dx.doi.org/10.1002/(SICI)1097-0061(199612)12:16<1607::AID-YEA70>3.0.CO;2-4.

13. **Avalos JL, Fink GR, Stephanopoulos G.** 2013. Compartmentalization of metabolic pathways in yeast mitochondria improves the production of branched-chain alcohols. Nat. Biotechnol. **31**:335–341. http://dx.doi.org/10.1038/nbt.2509.

14. **Chen Y, Daviet L, Schalk M, Siewers V, Nielsen J.** 2013. Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism. Metab. Eng. **15**:48–54. http://dx.doi.org/10.1016/j.ymben.2012.11.002.

15. **Kocharin K, Siewers V, Nielsen J.** 2013. Improved polyhydroxybutyrate production by *Saccharomyces cerevisiae* through the use of the phosphoketolase pathway. Biotechnol. Bioeng. **110**:2216–2224. http://dx.doi.org/10.1002/bit.24888.

16. **Krivoruchko A, Serrano-Amatriain C, Chen Y, Siewers V, Nielsen J.** 2013. Improving biobutanol production in engineered *Saccharomyces cerevisiae* by manipulation of acetyl-CoA metabolism. J. Ind. Microbiol. Biotechnol. **40**:1051–1056. http://dx.doi.org/10.1007/s10295-013-1296-0.

17. **Tang X, Feng H, Chen WN.** 2013. Metabolic engineering for enhanced fatty acids synthesis in *Saccharomyces cerevisiae*. Metab. Eng. **16**:95–102. http://dx.doi.org/10.1016/j.ymben.2013.01.003.

18. **Kozak BU, van Rossum HM, Benjamin KR, Wu L, Daran JM, Pronk JT, van Maris AJ.** 2014. Replacement of the *Saccharomyces cerevisiae* acetyl-CoA synthetases by alternative pathways for cytosolic acetyl-CoA synthesis. Metab. Eng. **21**:46–59. http://dx.doi.org/10.1016/j.ymben.2013.11.005.

19. **Snoep JL, de Graef MR, Westphal AH, de Kok A, Teixeira de Mattos MJ, Neijssel OM.** 1993. Differences in sensitivity to NADH of purified pyruvate dehydrogenase complexes of *Enterococcus faecalis*, *Lactococcus lactis*, *Azotobacter vinelandii* and *Escherichia coli*: implications for their activity *in vivo*. FEMS Microbiol. Lett. **114**:279–283. http://dx.doi.org/10.1111/j.1574-6968.1993.tb06586.x.

20. **Snoep JL, Westphal AH, Benen JA, Teixeira de Mattos MJ, Neijssel OM, de Kok A.** 1992. Isolation and characterisation of the pyruvate dehydrogenase complex of anaerobically grown *Enterococcus faecalis* NCTC 775. Eur. J. Biochem. **203**:245–250. http://dx.doi.org/10.1111/j.1432-1033.1992.tb19853.x.

21. **Canelas AB, van Gulik WM, Heijnen JJ.** 2008. Determination of the cytosolic free NAD/NADH ratio in *Saccharomyces cerevisiae* under steady-state and highly dynamic conditions. Biotechnol. Bioeng. **100**:734–743. http://dx.doi.org/10.1002/bit.21813.

22. **Cronan JE, Zhao X, Jiang Y.** 2005. Function, attachment and synthesis of lipoic acid in *Escherichia coli*. Adv. Microb. Physiol. **50**:103–146. http://dx.doi.org/10.1016/S0065-2911(05)50003-1.

23. **Hermes FA, Cronan JE.** 2013. The role of the Saccharomyces cerevisiae lipoate protein ligase homologue, *Lip3*, in lipoic acid synthesis. Yeast **30**:415–427.

24. **Martin N, Christensen QH, Mansilla MC, Cronan JE, de Mendoza D.** 2011. A novel two-gene requirement for the octanoyltransfer reaction of *Bacillus subtilis* lipoic acid biosynthesis. Mol. Microbiol. **80**:335–349. http://dx.doi.org/10.1111/j.1365-2958.2011.07597.x.

25. **De Virgilio C, Burckert N, Barth G, Neuhaus JM, Boller T, Wiemken A.** 1992. Cloning and disruption of a gene required for growth on acetate but not on ethanol: the acetyl-coenzyme A synthetase gene of *Saccharomyces cerevisiae*. Yeast **8**:1043–1051. http://dx.doi.org/10.1002/yea.320081207.

26. **van den Berg MA, Steensma HY.** 1995. *ACS2*, a *Saccharomyces cerevisiae* gene encoding acetyl-coenzyme A synthetase, essential for growth on glucose. Eur. J. Biochem. **231**:704–713. http://dx.doi.org/10.1111/j.1432-1033.1995.tb20751.x.

27. **de Jong-Gubbels P, van den Berg MA, Steensma HY, van Dijken JP, Pronk JT.** 1997. The *Saccharomyces cerevisiae* acetyl-coenzyme A synthetase encoded by the *ACS1* gene, but not the *ACS2*-encoded enzyme, is subject to glucose catabolite inactivation. FEMS Microbiol. Lett. **153**:75–81. http://dx.doi.org/10.1016/S0378-1097(97)00236-X.

28. **van den Berg MA, de Jong-Gubbels P, Kortland CJ, van Dijken JP, Pronk JT, Steensma HY.** 1996. The two acetyl-coenzyme A synthetases of *Saccharomyces cerevisiae* differ with respect to kinetic properties and transcriptional regulation. J. Biol. Chem. **271**:28953–28959. http://dx.doi.org/10.1074/jbc.271.46.28953.

29. **Luttik MA, Overkamp KM, Kötter P, de Vries S, van Dijken JP, Pronk JT.** 1998. The *Saccharomyces cerevisiae NDE1* and *NDE2* genes encode separate mitochondrial NADH dehydrogenases catalyzing the oxidation of cytosolic NADH. J. Biol. Chem. **273**:24529–24534. http://dx.doi.org/10.1074/jbc.273.38.24529.

30. **Henriksen P, Wagner SA, Weinert BT, Sharma S, Bacinskaja G, Rehman M, Juffer AH, Walther TC, Lisby M, Choudhary C.** 2012. Proteome-wide analysis of lysine acetylation suggests its broad regulatory scope in *Saccharomyces cerevisiae*. Mol. Cell. Proteomics **11**:1510–1522. http://dx.doi.org/10.1074/mcp.M112.017251.

31. **Lin YY, Lu JY, Zhang J, Walter W, Dang W, Wan J, Tao SC, Qian J, Zhao Y, Boeke JD, Berger SL, Zhu H.** 2009. Protein acetylation microarray reveals that NuA4 controls key metabolic target regulating gluconeogenesis. Cell **136**:1073–1084. http://dx.doi.org/10.1016/j.cell.2009.01.033.

32. **Lu JY, Lin YY, Sheu JC, Wu JT, Lee FJ, Chen Y, Lin MI, Chiang FT, Tai TY, Berger SL, Zhao Y, Tsai KS, Zhu H, Chuang LM, Boeke JD.** 2011. Acetylation of yeast AMPK controls intrinsic aging independently of caloric restriction. Cell **146**:969–979. http://dx.doi.org/10.1016/j.cell.2011.07.044.

33. **Shi L, Tu BP.** 2013. Acetyl-CoA induces transcription of the key G1 cyclin *CLN3* to promote entry into the cell division cycle in *Saccharomyces cerevisiae*. Proc. Natl. Acad. Sci. U. S. A. **110**:7318–7323. http://dx.doi.org/10.1073/pnas.1302490110.

34. **Weinert BT, Iesmantavicius V, Moustafa T, Schölz C, Wagner SA, Magnes C, Zechner R, Choudhary C.** 2014. Acetylation dynamics and stoichiometry in *Saccharomyces cerevisiae*. Mol. Syst. Biol. **10**:716. http://dx.doi.org/10.1002/msb.134766.

35. **Takahashi H, McCaffery JM, Irizarry RA, Boeke JD.** 2006. Nucleocytosolic acetyl-coenzyme A synthetase is required for histone acetylation and global transcription. Mol. Cell **23**:207–217. http://dx.doi.org/10.1016/j.molcel.2006.05.040.

36. **Huggett J, Dheda K, Bustin S, Zumla A.** 2005. Real-time RT-PCR normalisation; strategies and considerations. Genes Immun. **6**:279–284. http://dx.doi.org/10.1038/sj.gene.6364190.

37. **Marvin ME, Williams PH, Cashmore AM.** 2001. The isolation and characterisation of a *Saccharomyces cerevisiae* gene (*LIP2*) involved in the attachment of lipoic acid groups to mitochondrial enzymes. FEMS Microbiol. Lett. **199**:131–136. http://dx.doi.org/10.1111/j.1574-6968.2001.tb10663.x.

38. **Sulo P, Martin NC.** 1993. Isolation and characterization of *LIP5*. A lipoate biosynthetic locus of *Saccharomyces cerevisiae*. J. Biol. Chem. **268**:17634–17639.

39. **Schonauer MS, Kastaniotis AJ, Kursu VA, Hiltunen JK, Dieckmann CL.** 2009. Lipoic acid synthesis and attachment in yeast mitochondria. J. Biol. Chem. **284**:23234–23242. http://dx.doi.org/10.1074/jbc.M109.015594.

40. **Lian J, Si T, Nair NU, Zhao H.** 2014. Design and construction of acetyl-CoA overproducing *Saccharomyces cerevisiae* strains. Metab. Eng. **24**:139–149. http://dx.doi.org/10.1016/j.ymben.2014.05.010.

41. **Bates DL, Danson MJ, Hale G, Hooper EA, Perham RN.** 1977. Self-assembly and catalytic activity of the pyruvate dehydrogenase multienzyme complex of *Escherichia coli*. Nature **268**:313–316. http://dx.doi.org/10.1038/268313a0.

42. **Izard T, Sarfaty S, Westphal A, de Kok A, Hol WG.** 1997. Improvement of diffraction quality upon rehydration of dehydrated icosahedral *Enterococcus faecalis* pyruvate dehydrogenase core crystals. Protein Sci. **6**:913–915.

43. **Wilkinson KD, Williams CH, Jr..** 1981. NADH inhibition and NAD activation of *Escherichia coli* lipoamide dehydrogenase catalyzing the NADH-lipoamide reaction. J. Biol. Chem. **256**:2307–2314.

44. **Sonderegger M, Schümperli M, Sauer U.** 2004. Metabolic engineering of a phosphoketolase pathway for pentose catabolism in *Saccharomyces cerevisiae*. Appl. Environ. Microbiol. **70**:2892–2897. http://dx.doi.org/10.1128/AEM.70.5.2892-2897.2004.

45. **Entian KD, Kotter P.** 2007. 25 yeast genetic strain and plasmid collections. Methods Mol. Biol. **36:**629–666.

46. **Nijkamp JF, van den Broek M, Datema E, de Kok S, Bosman L, Luttik MA, Daran-Lapujade P, Vongsangnak W, Nielsen J, Heijne WH, Klaassen P, Paddon CJ, Platt D, Kotter P, van Ham RC, Reinders MJ, Pronk JT, de Ridder D, Daran JM.** 2012. *De novo* sequencing, assembly and analysis of the genome of the laboratory strain Saccharomyces cerevisiae CEN.PK113-7D, a model for modern industrial biotechnology. Microb. Cell Fact. **11:**36. http://dx.doi.org/10.1186/1475-2859-11-36.

47. **Verduyn C, Postma E, Scheffers WA, van Dijken JP.** 1992. Effect of benzoic acid on metabolic fluxes in yeasts: a continuous-culture study on the regulation of respiration and alcoholic fermentation. Yeast **8:**501–517. http://dx.doi.org/10.1002/yea.320080703.

48. **Pronk JT.** 2002. Auxotrophic yeast strains in fundamental and applied research. Appl. Environ. Microbiol. **68:**2095–2100. http://dx.doi.org/10.1128/AEM.68.5.2095-2100.2002.

49. **Roubos JA, v. Peij NNME.** 2008. A method for achieving improved polypeptide expression. International patent WO/2008/000632.

50. **Kuijpers NG, Solis-Escalante D, Bosman L, van den Broek M, Pronk JT, Daran JM, Daran-Lapujade P.** 2013. A versatile, efficient strategy for assembly of multi-fragment expression vectors in *Saccharomyces cerevisiae* using 60-bp synthetic recombination sequences. Microb. Cell Fact. **12:**47. http://dx.doi.org/10.1186/1475-2859-12-47.

51. **Gietz RD, Woods RA.** 2002. Transformation of yeast by lithium acetate/single-stranded carrier DNA/polyethylene glycol method. Methods Enzymol. **350:**87–96. http://dx.doi.org/10.1016/S0076-6879(02)50957-5.

52. **Gueldener U, Heinisch J, Koehler GJ, Voss D, Hegemann JH.** 2002. A second set of *loxP* marker cassettes for Cre-mediated multiple gene knockouts in budding yeast. Nucleic Acids Res. **30:**e23. http://dx.doi.org/10.1093/nar/30.6.e23.

53. **Oud B, Flores CL, Gancedo C, Zhang X, Trueheart J, Daran JM, Pronk JT, van Maris AJ.** 2012. An internal deletion in *MTH1* enables growth on glucose of pyruvate-decarboxylase negative, non-fermentative *Saccharomyces cerevisiae*. Microb. Cell Fact. **11:**131. http://dx.doi.org/10.1186/1475-2859-11-131.

54. **de Kok S, Yilmaz D, Suir E, Pronk JT, Daran JM, van Maris AJ.** 2011. Increasing free-energy (ATP) conservation in maltose-grown *Saccharomyces cerevisiae* by expression of a heterologous maltose phosphorylase. Metab. Eng. **13:**518–526. http://dx.doi.org/10.1016/j.ymben.2011.06.001.

55. **Guadalupe Medina V, Almering MJ, van Maris AJ, Pronk JT.** 2010. Elimination of glycerol production in anaerobic cultures of a *Saccharomyces cerevisiae* strain engineered to use acetic acid as an electron acceptor. Appl. Environ. Microbiol. **76:**190–195. http://dx.doi.org/10.1128/AEM.01772-09.

56. **Mashego MR, van Gulik WM, Vinke JL, Heijnen JJ.** 2003. Critical evaluation of sampling techniques for residual glucose determination in carbon-limited chemostat culture of *Saccharomyces cerevisiae*. Biotechnol. Bioeng. **83:**395–399. http://dx.doi.org/10.1002/bit.10683.

57. **Lowry OH, Rosebrough NJ, Farr AL, Randall RJ.** 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. **193:**265–275.

58. **Flikweert MT, van Dijken JP, Pronk JT.** 1997. Metabolic responses of pyruvate decarboxylase-negative *Saccharomyces cerevisiae* to glucose excess. Appl. Environ. Microbiol. **63:**3399–3404.

59. **Wenzel TJ, van den Berg MA, Visser W, van den Berg JA, Steensma HY.** 1992. Characterization of *Saccharomyces cerevisiae* mutants lacking the E1α subunit of the pyruvate dehydrogenase complex. Eur. J. Biochem. **209:**697–705. http://dx.doi.org/10.1111/j.1432-1033.1992.tb17338.x.

60. **Vogel O, Beikirch H, Muller H, Henning U.** 1971. The subunit structure of the *Escherichia coli* K-12 pyruvate dehydrogenase complex. The dihydrolipoamide transacetylase component. Eur. J. Biochem. **20:**169–178. http://dx.doi.org/10.1111/j.1432-1033.1971.tb01375.x.

61. **de Jonge LP, Buijs NA, ten Pierick A, Deshmukh A, Zhao Z, Kiel JA, Heijnen JJ, van Gulik WM.** 2011. Scale-down of penicillin production in *Penicillium chrysogenum*. Biotechnol. J. **6:**944–958. http://dx.doi.org/10.1002/biot.201000409.

62. **Lu P, Vogel C, Wang R, Yao X, Marcotte EM.** 2007. Absolute protein expression profiling estimates the relative contributions of transcriptional and translational regulation. Nat. Biotechnol. **25:**117–124. http://dx.doi.org/10.1038/nbt1270.

63. **Hazelwood LA, Walsh MC, Pronk JT, Daran JM.** 2010. Involvement of vacuolar sequestration and active transport in tolerance of *Saccharomyces cerevisiae* to hop iso-α-acids. Appl. Environ. Microbiol. **76:**318–328. http://dx.doi.org/10.1128/AEM.01457-09.

64. **Li H, Durbin R.** 2009. Fast and accurate short read alignment with Burrows-Wheeler transform. Bioinformatics **25:**1754–1760. http://dx.doi.org/10.1093/bioinformatics/btp324.

65. **Trapnell C, Williams BA, Pertea G, Mortazavi A, Kwan G, van Baren MJ, Salzberg SL, Wold BJ, Pachter L.** 2010. Transcript assembly and quantification by RNA-Seq reveals unannotated transcripts and isoform switching during cell differentiation. Nat. Biotechnol. **28:**-511–515. http://dx.doi.org/10.1038/nbt.1621.

66. **Knijnenburg TA, de Winde JH, Daran JM, Daran-Lapujade P, Pronk JT, Reinders MJ, Wessels LF.** 2007. Exploiting combinatorial cultivation conditions to infer transcriptional regulation. BMC Genomics **8:**25. http://dx.doi.org/10.1186/1471-2164-8-25.

67. **Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P.** 1992. Multifunctional yeast high-copy-number shuttle vectors. Gene **110:**119–122. http://dx.doi.org/10.1016/0378-1119(92)90454-W.

68. **Sikorski RS, Hieter P.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.