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Anaplerotic Role for Cytosolic Malic Enzyme in Engineered *Saccharomyces cerevisiae* Strains[▽]

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Malic enzyme catalyzes the reversible oxidative decarboxylation of malate to pyruvate and CO_2. The *Saccharomyces cerevisiae MAE1* gene encodes a mitochondrial malic enzyme whose proposed physiological roles are related to the oxidative, malate-decarboxylating reaction. Hitherto, the inability of pyruvate carboxylase-negative (Pyc⁻) *S. cerevisiae* strains to grow on glucose suggested that Mae1p cannot act as a pyruvate-carboxylating, anaplerotic enzyme. In this study, relocation of malic enzyme to the cytosol and creation of thermodynamically favorable conditions for pyruvate carboxylation by metabolic engineering, process design, and adaptive evolution, enabled malic enzyme to act as the sole anaplerotic enzyme in *S. cerevisiae*. The *Escherichia coli* NADH-dependent *sfcA* malic enzyme was expressed in a Pyc⁻ *S. cerevisiae* background. When *PDC2*, a transcriptional regulator of pyruvate decarboxylase genes, was deleted to increase intracellular pyruvate levels and cells were grown under a CO_2 atmosphere to favor carboxylation, adaptive evolution yielded a strain that grew on glucose (specific growth rate, $0.06 \pm 0.01 \ h^{-1}$). Growth of the evolved strain was enabled by a single point mutation (Asp336Gly) that switched the cofactor preference of *E. coli* malic enzyme from NADH to NADPH. Consistently, cytosolic relocalization of the native Mae1p, which can use both NADH and NADPH, in a *pyc1,2Δ pdc2Δ* strain grown under a CO_2 atmosphere, also enabled slow-growth on glucose. Although growth rates of these strains are still low, the higher ATP efficiency of carboxylation via malic enzyme, compared to the pyruvate carboxylase pathway, may contribute to metabolic engineering of *S. cerevisiae* for anaerobic, high-yield C_4-dicarboxylic acid production.

Malic enzyme is a ubiquitous enzyme that catalyzes the reversible oxidative decarboxylation of malate to pyruvate and CO_2, using either NAD^+ (EC 1.1.1.38-39) or $NADP^+$ (EC 1.1.1.40) as the redox cofactor. In eukaryotes, malic enzyme has been found in the cytosol and/or in mitochondria and, in plants, in the chloroplasts (20, 35). The reaction has a $\Delta G^{0'}$ of ca. $-8 \ kJ \ mol^{-1}$ in the decarboxylating direction (19) and, although both carboxylation and decarboxylation have been observed *in vitro*, substrate affinities and maximum reaction rates generally appear to favor the latter (5, 14, 34). Despite decades of study, the physiological function of malic enzyme often remains enigmatic. Proposed physiological roles for the decarboxylating reaction include provision of pyruvate, inorganic carbon, reducing power, and pH regulation (20, 26). Carboxylating roles for malic enzyme have also been suggested (25, 30), although *in vivo* evidence is sparse. The strongest indications for *in vivo* malic enzyme activity in the carboxylating direction come from experiments with engineered *Escherichia coli*, in which overexpression of malic enzyme improved succinate titers (17, 27, 28). However, interpretation of these results was complicated by the fact that these strains still expressed phospho-*enol*-pyruvate carboxylase, the main carboxylating enzyme in glucose-grown wild-type *E. coli* (2).

In the yeast *Saccharomyces cerevisiae*, malic enzyme is encoded by the *MAE1* gene (4). The mitochondrial Mae1 protein can use both NAD^+ and $NADP^+$ as an electron acceptor and exhibits a relatively high K_m toward malate (50 mM, or 17 mM in the presence of 0.5 mM phospho-*enol*-pyruvate [PEP]) (13). Also in *S. cerevisiae*, the physiological role of malic enzyme is still uncertain even though the enzyme has been shown to provide the pyruvate required for biosynthesis in pyruvate kinase-negative strains grown on ethanol (4). No kinetic data have been published for the carboxylating reaction, but the inability of pyruvate carboxylase-negative strains of *S. cerevisiae* to grow on glucose indicates that Mae1p cannot readily take over the anaplerotic role of pyruvate carboxylase (29). Consequently, the *in vivo* reaction catalyzed by Mae1p is often assumed to be irreversible in the decarboxylating direction (23).

In recent years, interest in the biotechnological production of C_4-dicarboxylic acids has intensified, as these acids are envisaged to become key building blocks in an increasingly "bio-based" chemical industry (24, 36). *S. cerevisiae* is an interesting production host for organic acids (1). Hitherto, attempts to engineer *S. cerevisiae* for malate (and succinate) production were based on replacement of the native alcoholic fermentation pathway by a malate fermentation pathway. To this end, pyruvate carboxylase, a cytosolically retargeted malate dehydrogenase and a heterologous malate transporter were (over)-expressed in a pyruvate decarboxylase-negative strain (38, 39). Although this approach yielded malate titers of up to 59 g liter⁻¹, ATP hydrolysis by pyruvate carboxylase precludes a net synthesis of ATP via this fermentative pathway. In practice, ATP yields are even expected to be negative due to energy requirements for dicarboxylic acid export (32). As a result, part of the glucose fed to the cultures has to be respired to provide

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TABLE 1. *S. cerevisiae* strains used in this study

Strain	Genotype
CEN.PK 113-7D	*MAT**a***, reference strain
IMK157 *ura3Δ*	*MAT**a** pyc1::kanMx pyc2::ILV2smr ura3-52*
IMK299	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52*
IMW012	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (cured); derived from IMY017
IMY007 empty vector	*MAT**a** pyc1::kanMx pyc2::ILV2smr ura3-52* (pUDC1)
IMY016	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (MB5573)
IMY017	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (MB5573*); evolved
IMY019	*MAT**a** pyc1::kanMx pyc2::ILV2smr ura3-52* (MB5573)
IMY024	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (MB5573*)
IMY025	*MAT**a** pyc1::kanMx pyc2::ILV2smr ura3-52* (pUDC10)
IMY026	*MAT**a** pyc1::kanMx pyc2::ILV2smr ura3-52* (pUDC9)
IMY027	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (pUDC10)
IMY028	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (pUDC9)
IMY029	*MAT**a** pyc1::kanMx pyc2::ILV2smr pdc2::loxP::hph::loxP ura3-52* (pUDC9); derived from IMY017
BY4741 *mae1Δ*	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mae1::kanMX*
IMY030	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mae1::kanMX* (pUDC1)
IMY031	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mae1::kanMX* (MB5573)
IMY032	*MAT**a** his3Δ1 leu2Δ0 met15Δ0 ura3Δ0 mae1::kanMX* (MB5573*)

ATP, which goes at the expense of the product yield on glucose. Improved understanding of the energetics and thermodynamics of C_3-C_4 carboxylation reactions is therefore not only of fundamental physiological interest but may also have industrial applications.

In the last step of glycolysis, pyruvate kinase transfers the phosphate group of PEP to ADP, producing pyruvate and ATP. In wild-type *S. cerevisiae* strains grown on glucose, anaplerosis occurs via pyruvate carboxylase, which converts pyruvate to oxaloacetate and consumes 1 ATP per carboxylation. As a result, the overall conversion of PEP to oxaloacetate is ATP neutral. However, if pyruvate kinase can be combined with malic enzyme, which converts pyruvate to malate without consuming ATP, PEP is converted to malate while producing net ATP. The same gain in ATP yield can be achieved by using phospho-*enol*-pyruvate carboxykinase (PEPCK), which converts PEP directly to oxaloacetate while producing ATP. Any reaction with an improved ATP yield has a lower thermodynamic driving force, since phosphorylation of ADP requires an input of free energy. That this can impose limits on metabolism is illustrated by a previous study, where PEPCK was shown to be able to function as an anaplerotic enzyme in *S. cerevisiae*, but only when concentrations of the substrates PEP and CO_2 were increased (40).

The goal of the present study was to investigate the requirements for an *in vivo* carboxylating role of malic enzyme in a pyruvate carboxylase-negative *S. cerevisiae* strain grown on glucose as the sole carbon source. Since in *S. cerevisiae* endogenous malic enzyme (Mae1p) is mitochondrial, while the pyruvate carboxylases (Pyc1p and Pyc2p) are cytosolic, various malic enzyme expression constructs were tested: (i) overexpression of the endogenous Mae1p, (ii) expression of a truncated form of Mae1p without the mitochondrial targeting sequence, and (iii) expression of the NADH-dependent malic enzyme from *E. coli*. In addition, the impact of the substrate concentrations of the malic enzyme reaction was tested by varying CO_2 concentrations and decreasing the activity of a pyruvate consuming reaction.

MATERIALS AND METHODS

Strains and maintenance. Strains constructed in the present study (Table 1) were derived from IMK157, an *S. cerevisiae* strain derived from the CEN.PK family (31), containing targeted deletions of *URA3* and the two pyruvate carboxylase genes *PYC1* and *PYC2* (40). Exceptions were strains IMY030 to IMY032, which originate from *S. cerevisiae* BY4741 *mae1Δ* (37). Strains were maintained on YPD (demineralized water; yeast extract [BD Difco], 10 g liter^{-1}; peptone [BD Difco], 20 g liter^{-1}; glucose, 20 g liter^{-1}) or, for strains carrying plasmids, on synthetic stock medium (demineralized water; KH$_2$PO$_4$, 3 g liter^{-1}; MgSO$_4$, 0.5 g liter^{-1}; L-aspartic acid [from a filter-sterilized 7.4-g liter^{-1} solution set to pH 6 with KOH], 6.6 g liter^{-1}; K$_2$SO$_4$, 6.6 g liter^{-1}; glucose, 20 g liter^{-1}; trace elements and filter-sterilized vitamins) (33). Culture stocks, prepared from shake flask cultures by the addition of glycerol (20% [vol/vol]), were stored at −80°C in 1-ml aliquots. Incubations were performed at 30°C, and shake flasks were kept in orbital shakers at 200 rpm.

Plasmid construction and transformation. Plasmid pUG-hphNT1 (Table 2) was constructed by replacing the *kanMX* marker in pUG6 (15) with the *hphNT1*

TABLE 2. Plasmids used in this study

Plasmid	Characteristics	Reference
pFA6a-hphNT1	Template for *hphNT1*	18
pUG6	Template for *loxP-kanMX-loxP* deletion cassette	15
pUG-hphNT1	Template for *loxP-hphNT1-loxP* deletion cassette	This study
p416-GPD	Centromeric yeast vector, *URA3*, P$_{TDH3}$-T$_{CYC1}$	22
MB5573	Centromeric yeast vector, *URA3*, P$_{TDH3}$-*E. coli sfcA*-T$_{CYC1}$	This study
MB5573*	Centromeric yeast vector, *URA3*, P$_{TDH3}$-*E. coli sfcA*-T$_{CYC1}$, isolated from strain IMY017	This study
pUDC1	Centromeric yeast vector, *URA3*, P$_{TDH3}$-T$_{CYC1}$	40
pUDC9	Centromeric yeast vector, *URA3*, P$_{TDH3}$-*sMAE1*-T$_{CYC1}$	This study
pUDC10	Centromeric yeast vector, *URA3*, P$_{TDH3}$-*MAE1*-T$_{CYC1}$	This study

TABLE 3. Oligonucleotides used in this study

Oligonucleotide	Sequence (5′–3′)
5′-PDC2-S1	ACGCAACTTGAATTGGCAAAATGGGCTTATGAGACGTTCCCAGCTGAAGCTTCGTACGC
3′-PDC2-S2	AGCCTGTGTTACCAGGTAAGTGTAAGTTATTAGAGTCTGGGCATAGGCCACTAGTGGATCTG
5′-MAE1-XbaI	CGACGGATTCTAGAGGTTATGCTTAGAACCAGACTATCCG
5′-sMAE1-XbaI	ACCATCTAGAATGTGGCCTATTCAGCAATCGCG
3′-MAE1-SalI	GTTAGTCGACCTACAATTGGTTGGTGTGCACC

marker (conferring hygromycin B resistance) from pFA6a-hphNT1 (18) using the SacI and BglII restriction sites. The *PDC2* deletion cassette was prepared by PCR using pUG-hphNT1 with primers 5′-PDC2-S1 and 3′-PDC2-S2 (Table 3) (10). Transformants of strain IMK157 were selected on YP agar containing ethanol (3% [vol/vol]), glycerol (2% [vol/vol]), and hygromycin B (200 mg liter^{-1}). A single colony isolate, for which deletion of *PDC2* was confirmed by PCR, was named IMK299.

A synthetic gene, consisting of *E. coli* *sfcA* malic enzyme (also known as *maeA*) with flanking SpeI and XhoI restriction sites, was inserted into p416-GPD (22) digested with XbaI and XhoI (synthesis and codon optimization for expression in *S. cerevisiae* were performed by Blue Heron Biotechnology, Bothell, WA). The resulting plasmid was named MB5573 (Table 2). *S. cerevisiae* *MAE1* and the truncated version *sMAE1* (21) were amplified from CEN.PK 113-7D genomic DNA with primers 5′-MAE1-XbaI or 5′-sMAE1-XbaI and 3′-MAE1-SalI (Table 3). After digestion with XbaI and SalI, the PCR products were inserted into p416-GPD via the XbaI and XhoI sites, resulting in plasmids pUDC9 (expressing *sMAE1*) and pUDC10 (expressing *MAE1*) (Table 2). Correct PCR amplification was verified by sequencing, performed by BaseClear, Netherlands.

Yeast transformations were performed as described previously (7). Transformants were selected on synthetic glucose medium agar plates without uracil and with aspartate as the nitrogen source. Histidine (25 mg liter^{-1}), leucine (30 mg liter^{-1}), and methionine (25 mg liter^{-1}) were added as required. Transformations were verified by PCR.

Plate cultivation. Solid media were prepared by addition of 20 g of Bacto agar liter^{-1}. Apart from the nitrogen source [either 4 g of aspartate or 2 g of (NH$_4$)$_2$SO$_4$ liter^{-1}] and the addition of Tween 80 (0.42 g liter^{-1}) and ethanol-dissolved ergosterol (10 mg liter^{-1}), added to allow for growth under anaerobic conditions, solid synthetic media were identical to the synthetic stock culture medium. Gas-tight jars with 0.2 bar of overpressure were used for incubation of agar plates under a CO$_2$ atmosphere.

Batch cultivation. Batch cultivation was performed at 30°C in 2-liter bioreactors (Applikon, Schiedam, Netherlands) at a working volume of 1 liter. The pH was controlled by automatic addition of 2 M KOH. Bioreactors were sparged with 250 ml of CO$_2$ per min and stirred at 800 rpm. The medium was identical to the synthetic stock culture medium, except for the replacement of the aspartic acid and K$_2$SO$_4$ by 5 g of (NH$_4$)$_2$SO$_4$ liter^{-1} and the addition of 0.15 ml of silicon antifoam (BDH, Poole, England) liter^{-1} to control foaming. Tween 80 (0.42 g liter^{-1}) and ethanol-dissolved ergosterol (10 mg liter^{-1}) were added to the medium to allow for growth under anaerobic conditions. For growth rate measurements, the initial optical density at 660 nm (OD$_{660}$) after inoculation was ≤0.1. Maximum specific growth rates were determined for duplicate cultures (errors are given as deviations from the mean) and based on the OD$_{660}$. Preculture shake flasks with synthetic stock medium were inoculated with 1-ml aliquots of frozen stock culture. Cells from exponentially growing shake-flask precultures were washed twice with demineralized water and used to inoculate batch cultures.

Selection of IMY017. Nitrogen-limited medium for the selection of strain IMY017 was based on the synthetic stock culture medium, with the following modifications: medium contained 1 g of (NH$_4$)$_2$SO$_4$ and 5.3 g of K$_2$SO$_4$ liter^{-1}, as well as 30 g of glucose liter^{-1}, 0.15-ml liter^{-1} of silicon antifoam (BDH, Poole, England) to control foaming, Tween 80 (0.42 g liter^{-1}), and ethanol-dissolved ergosterol (10 mg liter^{-1}) to allow for growth under anaerobic conditions. Medium was added via a peristaltic pump, and the working volume was kept constant by means of a conductive level sensor.

Plasmid isolation and curing. The plasmid from IMY017 was isolated with a Zymoprep II yeast plasmid miniprep kit and amplified in *E. coli*. Sequencing of the recovered plasmid, which was designated MB5573* (Table 2), was performed by BaseClear. Plasmid loss in strain IMY017 was achieved by growth under nonselective conditions on liquid YPD. After several serial transfers, the culture was plated on YPD agar. A single colony isolate, which displayed uracil auxotrophy and for which plasmid loss was supported by PCR, was named IMW012 (Table 1).

Metabolite analysis. Extracellular concentrations of glucose and ethanol were determined by high-performance liquid chromatography, using a Bio-Rad Aminex HPX-87H column eluted with 5 mM H$_2$SO$_4$ at a flow rate of 0.6 ml min^{-1} and kept at 60°C, coupled to a Waters 2410 refractive index detector.

Preparation of cell extracts. Samples for enzyme activity measurements were obtained from shake flask cultures on synthetic stock medium, with glucose and aspartate, or 3% (vol/vol) ethanol as the carbon source(s). Cells, harvested in the exponential phase before depletion of either glucose or ethanol, were centrifuged, washed, and resuspended in potassium phosphate buffer (10 mM [pH 7.5], with 2 mM EDTA) and stored at −20°C (each sample contained ~60 mg biomass dry weight). Cell extracts for isocitrate lyase, pyruvate decarboxylase, and phospho-*enol*-pyruvate carboxykinase were prepared as described earlier (41). For malic enzyme, cell extracts were prepared in 50 mM Tris-HCl buffer (pH 7.5) containing 1 mM dithiothreitol and 2 mM MgCl$_2$ and dialyzed against this buffer for 4 h at 4°C using 3 ml 10K MWCO Slide-A-Lyzer Dialysis Cassettes (Thermo Scientific).

Enzyme activity assays. Activities of isocitrate lyase, pyruvate decarboxylase and phospho-*enol*-pyruvate carboxykinase were determined as previously described (9, 11). As similar assay mixtures have been used to determine *in vitro* malic enzyme activities for *S. cerevisiae* and *E. coli* (4, 5), malic enzyme activities were determined as described for *S. cerevisiae* (4) with either 0.4 mM NAD$^+$ or NADP$^+$ as the redox cofactor. Malic enzyme activity was measured in the decarboxylating direction to avoid interference with pyruvate decarboxylase and alcohol dehydrogenase. Values for V_{max} and K_m were determined with nonlinear fits, assuming Michaelis-Menten kinetics (using GraphPad Prism 4).

RESULTS

Expression of *E. coli* NAD$^+$-dependent malic enzyme is insufficient to rescue C$_4$ auxotrophy of Pyc$^-$ *S. cerevisiae*. In wild-type *S. cerevisiae* grown on glucose, pyruvate carboxylase functions as the sole anaplerotic enzyme (6, 29), replenishing the intracellular pools of C$_4$ acids. To test whether malic enzyme can assume this anaplerotic role in pyruvate carboxylase-negative (Pyc$^-$) *S. cerevisiae*, a codon-optimized version of the *E. coli* NAD$^+$-dependent malic enzyme, *sfcA* (also known as *maeA*), was overexpressed in the *pyc1Δ pyc2Δ* strain IMK157, resulting in strain IMY019. Compared to the Pyc$^-$ empty vector control strain IMY007, overexpression of the *E. coli* malic enzyme increased *in vitro* NAD$^+$-dependent malic enzyme activity by circa 8-fold to 0.49 ± 0.03 mmol min^{-1} g of protein^{-1} (Table 4). However, strain IMY019 showed no growth on synthetic medium agar plates with glucose as the sole carbon source, even when cultures were incubated under a CO$_2$ atmosphere to thermodynamically favor pyruvate carboxylation (Fig. 1).

To test whether increased intracellular substrate concentrations would allow for an anaplerotic flux through malic enzyme, an attempt was made to increase intracellular pyruvate levels by deleting *PDC2*, which encodes a positive regulator of the pyruvate decarboxylase (Pdc) isozymes *Pdc1* and *Pdc5* (16). Pyruvate decarboxylases convert pyruvate to acetaldehyde, a crucial step in the formation of ethanol. Deletion of *PDC2* has previously been shown to result in a 3- to 4-fold reduction of Pdc activity in aerobic glucose-limited chemostat cultures, and

TABLE 4. *In vitro* whole-cell enzyme activities

Strain	μ^{max} (h^{-1})	Mean enzyme activity (mmol min^{-1} g of $protein^{-1}$) $\pm$ SD[a]				
		ME-NAD	ME-NADP	Pdc	PEPCK	Icl
CEN.PK 113-7D	0.30 $\pm$ 0.01	0.04 $\pm$ 0.00	0.03 $\pm$ 0.00	1.5 $\pm$ 0.2	0.04 $\pm$ 0.01	ND
CEN.PK 113-7D on ethanol	NA	0.09 $\pm$ 0.02	0.02 $\pm$ 0.00	0.2 $\pm$ 0.0	0.44 $\pm$ 0.05	0.22 $\pm$ 0.03
IMY007 (*pyc1,2Δ*, empty vector)	NG	0.06 $\pm$ 0.00	0.03 $\pm$ 0.01	1.9 $\pm$ 0.3	0.04 $\pm$ 0.00	ND
IMY019 (*pyc1,2Δ*, *E. coli sfcA*)	NG	0.49 $\pm$ 0.03	0.03 $\pm$ 0.00	1.6 $\pm$ 0.1	0.04 $\pm$ 0.00	ND
IMY016 (*pyc1,2Δ pdc2Δ*, *E. coli sfcA*)	NG	1.03 $\pm$ 0.07	0.03 $\pm$ 0.00	0.7 $\pm$ 0.0	0.04 $\pm$ 0.01	ND
IMY017 (*pyc1,2Δ pdc2Δ* evolved, mutated *E. coli sfcA*)	0.06 $\pm$ 0.01	0.12 $\pm$ 0.01	0.39 $\pm$ 0.02	0.7 $\pm$ 0.0	0.04 $\pm$ 0.02	ND
IMY027 (*pyc1,2Δ pdc2Δ*, ↑*MAE1*)	NG	0.30 $\pm$ 0.03	0.34 $\pm$ 0.04	0.5 $\pm$ 0.0	0.04 $\pm$ 0.00	ND
IMY028 (*pyc1,2Δ pdc2Δ*, ↑*sMAE1*)	0.012 $\pm$ 0.000	0.17 $\pm$ 0.01	0.18 $\pm$ 0.01	0.7 $\pm$ 0.0	0.04 $\pm$ 0.00	ND
IMY029 (*pyc1,2Δ pdc2Δ*, ↑*sMAE1*), IMY017 background	0.019 $\pm$ 0.003	0.18 $\pm$ 0.02	0.19 $\pm$ 0.02	1.3 $\pm$ 0.0	0.04 $\pm$ 0.00	ND

[a] Abbreviations: ME-NAD; malic enzyme (with NAD^+); ME-NADP, malic enzyme (with $NADP^+$); Pdc, pyruvate decarboxylase; PEPCK, phospho-*enol*-pyruvate carboxykinase; Icl, isocitrate lyase. Yeast strains were grown on synthetic medium with glucose and aspartate unless indicated otherwise. Errors are deviations from mean (for each condition two shake flask cultures were run). Also indicated are the maximum specific growth rates (μ^{max}) on glucose under a CO_2 atmosphere in batch cultures. NA, not applicable; ND, not detectable (<0.005 mmol min^{-1} g of $protein^{-1}$); NG, no growth (tested on plates).

in substantial production of pyruvate during batch cultivation on glucose (10). In the present study, *PDC2* was deleted in strain IMK157, yielding strain IMK299. Subsequent overexpression of the *E. coli sfcA* malic enzyme in the *pyc1,2Δ pdc2Δ* strain IMK299 resulted in strain IMY016 (Table 1). Although a >2-fold reduction in pyruvate decarboxylase activity was observed in cell extracts relative to the parental strain IMY019 (Table 4), strain IMY016 showed no growth on glucose, regardless of whether an air or CO_2 atmosphere was applied (Fig. 1).

Evolutionary engineering for increased anaplerotic flux. To select for mutants with an increased anaplerotic flux through malic enzyme, evolutionary engineering was used, a strategy that previously proved successful for enabling anaplerosis in *S. cerevisiae* via PEPCK. After precultivation on glucose and aspartate, strain IMY016 was inoculated at an initial OD660 of circa 0.5 in a CO_2-sparged batch bioreactor, containing synthetic medium with glucose as the sole carbon source. After a week of incubation without growth, aspartate was pulsed to allow the (viable) biomass concentration to increase. Subsequent nitrogen-limited chemostat cultivation on glucose medium at a dilution rate of 0.05 h^{-1} resulted in partial washout, and feeding was stopped. During the following batch phase, growth was observed. After two additional washout-batch cycles on medium with glucose as the sole carbon source, a specific growth rate of 0.06 h^{-1} was reached. At the end of the
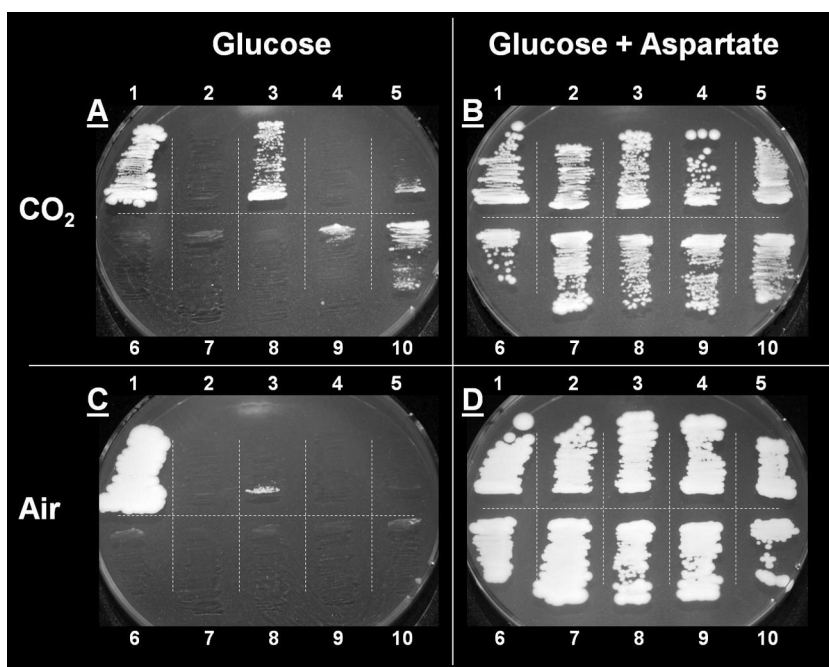

FIG. 1. Synthetic medium agar plates, incubated for 14 days under an atmosphere of either 1.2 bar CO_2 (plates A and B) or air (plates C and D). Plates contained glucose, Tween 80 and ergosterol and, as the nitrogen source, either ammonium sulfate (plates A and C) or aspartate (plates B and D). Strains: 1, CEN.PK 113-7D (wild-type reference); 2, IMY016 (*pyc1,2Δ pdc2Δ*, *E. coli sfcA*); 3, IMY017 (*pyc1,2Δ pdc2Δ* evolved, mutated *E. coli sfcA*); 4, IMY019 (*pyc1,2Δ*, *E. coli sfcA*); 5, IMY024 (*pyc1,2Δ pdc2Δ*, mutated *E. coli sfcA*); 6, IMY025 (*pyc1,2Δ*, ↑*MAE1*); 7, IMY026 (*pyc1,2Δ*, ↑*sMAE1*); 8, IMY027 (*pyc1,2Δ pdc2Δ*, ↑*MAE1*); 9, IMY028 (*pyc1,2Δ pdc2Δ*, ↑*sMAE1*); 10, IMY029 (*pyc1,2Δ pdc2Δ* evolved, ↑*sMAE1*). *sMAE1* is a truncated, cytosolically retargeted version of *S. cerevisiae MAE1*.

TABLE 5. Kinetic parameters of the original allele of *E. coli* *sfcA* NADH-dependent malic enzyme and of a mutated allele obtained by adaptive evolution in yeast, with either NAD$^+$ or NADP$^+$ as the redox cofactor

Strain	Kinetic enzyme parameters (mean ± SD)[a]			
	NAD$^+$		NADP$^+$	
	V_{max} (mmol min^{-1} g of protein^{-1})	K_m (mM)	V_{max} (mmol min^{-1} g of protein^{-1})	K_m (mM)
IMY031 (*mae1Δ, E. coli sfcA*)	1.8 ± 0.2	0.14 ± 0.05	0.6 ± 0.3	8.7 ± 7.3
IMY032 (*mae1Δ, mutated E. coli sfcA*)	1.0 ± 0.2	3.9 ± 1.9	0.7 ± 0.1	0.23 ± 0.08

[a] Measurements were performed in whole-cell extracts of *S. cerevisiae* strains grown on synthetic medium with glucose and aspartate. Ranges indicate 95% confidence intervals (for each strain, enzyme assays were performed on cell extracts from two independent shake flasks).

bioreactor cultivation a culture sample was taken and incubated on synthetic medium agar with glucose as the sole carbon source. Growth on plates was now observed, but only when plates were incubated under a CO_2 atmosphere. This was consistent with an anaplerotic role of malic enzyme and indicated that growth did not result from cells with a derepressed glyoxylate cycle (3), for which CO_2 is not a substrate. A single colony isolate from the evolved culture, designated IMY017 (Fig. 1), showed a representative maximum specific growth rate of 0.06 ± 0.01 h^{-1} in CO_2-sparged batch cultures on glucose. At a specific growth rate of 0.06 h^{-1}, a single gain-of-function mutant cell would be able to grow to a stationary phase culture in the 1-liter bioreactor (corresponding to approximately 35 generations) within 2 weeks. This time span is well within the 1-month duration of the selective cultivation.

Reverse engineering: a switch in cofactor specificity of *E. coli* malic enzyme. To identify the basis of the changed phenotype, the *in vitro* enzyme activities of malic enzyme, pyruvate decarboxylase, PEPCK, and isocitrate lyase were determined in cell extracts of strain IMY017. Consistent with the strong CO_2 dependence observed for growth on glucose (Fig. 1), no activity was found for isocitrate lyase, confirming that the glyoxylate cycle, which is normally repressed during batch cultivation on glucose, did not act as an anaplerotic pathway in the evolved strain. The PEPCK activity remained low at 0.04 ± 0.02 mmol min^{-1} g of protein^{-1}, while no further decrease in Pdc activity was observed compared to IMY016 (Table 4). However, large changes were observed for malic enzyme. Whereas NAD$^+$-dependent malic enzyme activity had dropped over 8-fold to 0.12 ± 0.01 mmol min^{-1} g of protein^{-1}, NADP$^+$-dependent activity had increased over 10-fold to 0.39 ± 0.02 mmol min^{-1} g of protein^{-1} (Table 4).

The enzyme activity measurements provided strong indications that the new phenotype was primarily due to changes to *E. coli* malic enzyme. Indeed, when the plasmid from IMY017, named MB5573*, was retransformed to IMK299, the resulting strain immediately showed growth on glucose under a CO_2 atmosphere (strain IMY024, Fig. 1). Growth was slower than for IMY017, suggesting that additional beneficial mutations had occurred in IMY017 on a genomic level. No growth was observed when the original MB5573 plasmid or an empty plasmid were retransformed to a cured IMY017, or when the MB5573* plasmid isolated from IMY017 was transformed to strain IMK157, which still carried an intact copy of *PDC2* (data not shown).

Subsequent sequencing of the *sfcA* open reading frame, as well as its *TDH3* promoter and *CYC1* terminator, in the MB5573* strain uncovered a single A1007G point mutation resulting in an Asp336Gly amino acid change. This mutation lies in the NAD domain of *sfcA* and is close to the 311- to 319-amino-acid sequence that is predicted to be the NAD(P)-binding site (5). Interestingly, sequencing of the plasmid from an independently evolved single colony isolate, which like strain IMY017 showed growth on glucose but was not further investigated due to a low specific growth rate on glucose of 0.02 ± 0.00 h^{-1}, uncovered a G1006A point mutation, resulting in a change of the same amino acid residue (in this case an Asp336Asn substitution).

To test whether the A1007G mutation had indeed changed the cofactor specificity and to avoid interference of the endogenous Mae1p in enzyme assays, the original and mutated *E. coli* malic enzyme were introduced in a *mae1* knockout strain from the *Saccharomyces* Genome Deletion Project (37) (Table 1). Although PCR confirmed the deletion of *mae1*, low background activities were still found in the malic enzyme assay for a *mae1* deletion strain carrying an empty vector (strain IMY030). However, at ca. 5% of the activities measured for the strains carrying MB5573 (IMY031) or MB5573* (IMY032), this did not significantly influence the characterization of the *E. coli* malic enzyme. Interestingly, the A1007G point mutation in *sfcA* resulted in a 2-fold lower V_{max} on NAD$^+$, and a slightly increased V_{max} on NADP$^+$ (Table 5). However, a far more striking effect was observed for the cofactor substrate saturation constants. In the mutated *E. coli* malic enzyme, the K_m for NAD$^+$ had decreased ~30-fold, while the K_m for NADP$^+$ was improved by approximately the same factor (Table 5). Combined, these changes effected a drastic switch in cofactor specificity.

An anaplerotic role for cytosolically targeted *S. cerevisiae* Mae1p. After having established the importance of cofactor specificity in the anaplerotic role of the *E. coli* enzyme, replacement of the anaplerotic role of pyruvate carboxylase by alleles of the native yeast malic enzyme, encoded by *MAE1*, was investigated. Interestingly, the endogenous malic enzyme already has the potential to use NADPH as the cofactor, but is, in contrast to the heterologously expressed *E. coli* enzyme, targeted to the mitochondrion. Therefore, both a full copy of *MAE1* and a truncated version lacking the mitochondrial targeting sequence, named *sMAE1* (21), were overexpressed in Pyc$^-$ *S. cerevisiae*. No growth was discernible on synthetic glucose medium for the Pyc$^-$ strain IMK157 (over)expressing *MAE1* (strain IMY025) or *sMAE1* (IMY026), and for the *pyc1,2Δ pdc2Δ* strain IMK299 overexpressing *MAE1* (IMY027). However, colonies were obtained for the *pyc1,2Δ*

pdc2Δ strain IMK299 expressing the cytosolic *sMAE1* (IMY028, see Fig. 1). This strain showed a maximum specific growth rate of 0.012 ± 0.000 h^{-1} on glucose in CO_2-sparged batch cultures.

The characterization of the evolved strain IMY017, which expresses a mutated copy of the *E. coli sfcA* gene, suggested that changes in the yeast genetic background, although not essential for growth, contributed to its improved growth. To test whether these unidentified genomic changes would also benefit a strain expressing the truncated *S. cerevisiae* malic enzyme, *sMAE1* was expressed in IMY017 after its original plasmid had been cured. Indeed, growth of the resulting strain IMY029 was faster on glucose plates (Fig. 1) and in CO_2-sparged batch cultures, where a specific growth rate of 0.019 ± 0.003 h^{-1} was observed. The relatively low growth rate of this *sMAE1*-based strain (the *E. coli sfcA*-based strain IMY017 showed a maximum specific growth rate of 0.06 ± 0.01 h^{-1}) might in part be explained by a lower NADP$^+$-dependent malic enzyme activity for strain IMY029 compared to strain IMY017 (0.19 ± 0.02 versus 0.39 ± 0.02 mmol min^{-1} g of protein^{-1}; see Table 4).

DISCUSSION

This study shows that, in addition to a derepressed glyoxylate cycle (3), heterologous PEP carboxylase (12), and PEPCK (40), malic enzyme can assume the anaplerotic role in *S. cerevisiae* during growth on glucose that is normally fulfilled by pyruvate carboxylase (6, 29). This is substantiated by the fact that isocitrate lyase activity in the Pyc$^-$ strains remained below the detection level, growth depended on high CO_2 levels, which would only benefit anaplerotic routes involving carbon fixation, and, also in the evolved strains, growth required the overexpression of (cytosolic) malic enzyme. A significant carboxylating flux through PEPCK is not expected, since PEPCK activities were not increased after evolution, and *in vitro* enzyme activities were only a fraction of those found in PEPCK-anaplerotic strains (0.04 versus 0.29 to 0.87 mmol min^{-1} g of protein^{-1}; see reference 40). Similarly, the low expression levels of endogenous *MAE1* and mitochondrial localization of Mae1p make it unlikely that malic enzyme made a significant contribution to the anaplerotic flux in the earlier-characterized PEPCK-expressing *S. cerevisiae* strains (40). The present study has shown that a cytosolic localization is crucial for enabling an anaplerotic role of malic enzyme, which might be explained by less favorable conditions for carboxylation in the mitochondrial matrix, an inability to generate mitochondrial NAD(P)H, and/or limitations in the transport of C_4 acids from the mitochondria to the cytosol.

NADPH as a preferred cofactor. The NAD$^+$-specific *E. coli sfcA* malic enzyme was chosen based on several grounds: (i) as a prokaryotic protein, *sfcA* lacks an intracellular targeting sequence, and thus can be expected to be cytosolically expressed in *S. cerevisiae*; (ii) previous studies on malic enzyme as a carboxylating enzyme in *E. coli* also targeted *sfcA* (17, 27); and (iii) if malic enzyme would be used for the high-yield production of C_4-dicarboxylic acids, the use of NADH instead of NADPH would simplify the balancing of cofactors (e.g., avoiding the need for transhydrogenase activity for the production of malate and fumarate, which can be produced redox neu-

trally from glucose). However, the Pyc$^-$ *S. cerevisiae* only grew when expressing either the cytosolically relocalized sMAE1p, which can use both NADH and NADPH, or a mutated *sfcA* allele with a strongly improved affinity for NADPH. This indicates that, in the tested strains, anaplerosis via malic enzyme depended on the use of NADPH for reductive pyruvate carboxylation.

The Asp336Gly mutation clearly resulted in a near complete switch of the cofactor specificity from NAD$^+$ to NADP$^+$ in *E. coli* malic enzyme. This preferential use of NADPH by malic enzyme in this carboxylating role might be explained by the thermodynamics of the catalyzed reaction. The dependency of growth on deletion of *PDC2*, which probably increased the cytosolic pyruvate concentration, and on incubation under a CO_2 atmosphere, both indicate that this reaction is likely to be close to thermodynamic equilibrium when operating in the carboxylating direction. Although few measurements are available on cytosolic redox factor concentrations in *S. cerevisiae* (8), whole-cell measurements showed intracellular ratios of NADPH/NADP$^+$ that are higher than those of NADH/NAD$^+$ (21). If this is representative for the cofactor distribution in the cytosol, the use of NADPH would increase the driving force of carboxylation by cytosolic malic enzyme.

Malic enzyme for C_4 acid production by *S. cerevisiae*. Like PEPCK, malic enzyme offers the prospect of metabolic engineering of *S. cerevisiae* for C_4-dicarboxylic acid production from glucose under anaerobic conditions (40). The higher ATP yield of carboxylation might eliminate the need to respire part of the glucose for ATP generation, thereby increasing product yields. In view of the relatively low growth rates of the current strains (0.06 h^{-1} with the mutated *E. coli* malic enzyme versus 0.14 h^{-1} for PEPCK-anaplerotic strains and 0.30 h^{-1} for the pyruvate carboxylase-positive reference strain [40]), the first priority will be to increase the *in vivo* rates of pyruvate carboxylation by malic enzyme. Initially, sequential batch cultivation can be used to select for faster growth and a higher anaplerotic flux. Further increases in the carboxylating fluxes through malic enzyme or PEPCK might be achieved by improving substrate availability and reducing the flux to ethanol. Such an approach previously proved successful in engineering a pyruvate carboxylase-overexpressing *S. cerevisiae* strain, which was unable to produce ethanol due to deletion of the pyruvate decarboxylase genes, for malate and succinate production (38, 39). The increased availability of pyruvate in pyruvate decarboxylase-negative strains can also improve the carboxylation through malic enzyme. As an additional benefit, when ATP generation becomes dependent on the production (and export) of C_4-dicarboxylic acids, this might allow for evolutionary engineering for an improved flux through malic enzyme (40).

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