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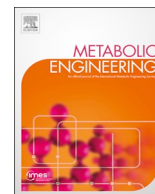
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Functional expression of a bacterial α -ketoglutarate dehydrogenase in the cytosol of *Saccharomyces cerevisiae*



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ABSTRACT

Efficient production of fuels and chemicals by metabolically engineered micro-organisms requires availability of precursor molecules for product pathways. In eukaryotic cell factories, heterologous product pathways are usually expressed in the cytosol, which may limit availability of precursors that are generated in other cellular compartments. In *Saccharomyces cerevisiae*, synthesis of the precursor molecule succinyl-Coenzyme A is confined to the mitochondrial matrix. To enable cytosolic synthesis of succinyl-CoA, we expressed the structural genes for all three subunits of the *Escherichia coli* α -ketoglutarate dehydrogenase (α KGDH) complex in *S. cerevisiae*. The *E. coli* lipoic-acid scavenging enzyme was co-expressed to enable cytosolic lipoylation of the α KGDH complex, which is required for its enzymatic activity. Size-exclusion chromatography and mass spectrometry indicated that the heterologously expressed α KGDH complex contained all subunits and that its size was the same as in *E. coli*. Functional expression of the heterologous complex was evident from increased α KGDH activity in the cytosolic fraction of yeast cell homogenates. *In vivo* cytosolic activity of the α KGDH complex was tested by constructing a reporter strain in which the essential metabolite 5-aminolevulinic acid could only be synthesized from cytosolic, and not mitochondrial, succinyl-CoA. To this end *HEMI*, which encodes the succinyl-CoA-converting mitochondrial enzyme 5-aminolevulinic acid (ALA) synthase, was deleted and a bacterial ALA synthase was expressed in the cytosol. In the resulting strain, complementation of ALA auxotrophy depended on activation of the α KGDH complex by lipoic acid addition. Functional expression of a bacterial α KGDH complex in yeast represents a vital step towards efficient yeast-based production of compounds such as 1,4-butanediol and 4-aminobutyrate, whose product pathways use succinyl-CoA as a precursor.

1. Introduction

Production of industrially relevant compounds with engineered microorganisms relies on introduction of heterologous product pathways as well as on changing the expression and/or sequences of native genes. Selection of a suitable production organism is based on several criteria, including native ability to synthesize the compound of interest, robustness to industrial conditions, ability to excrete the product and genetic accessibility. *Saccharomyces cerevisiae* is a popular microbial platform for production of low-molecular-weight compounds. Recent advances in genome editing technology have helped to expand its product spectrum from simple products such as succinic acid (Jansen

et al., 2017) to complex pharmaceutical molecules such as artemisinic acid (Ro et al., 2006) and opioids (Galanie et al., 2015). *S. cerevisiae* is used for industrial production of, among others, farnesene, resveratrol, artemisinic acid and artificial sweeteners (Ekas et al., 2019). In *S. cerevisiae*, heterologous product pathways are usually expressed in the cytosol. Since, as in other eukaryotes, metabolism in *S. cerevisiae* is highly compartmentalized, the intracellular localization of metabolic precursors is a key aspect in the design and construction of engineered strains (Avalos et al., 2013; van Rossum et al., 2016b).

In *S. cerevisiae*, succinyl-CoA is synthesized in the mitochondria, where it is used as a substrate for 5-aminolevulinic acid synthase, which catalyses the first committed step in heme biosynthesis (Hoffman et al.,

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2003). Succinyl-CoA is an important precursor molecule in microbial biotechnology, as it can be reduced to succinate semialdehyde and subsequently to 4-hydroxybutyrate (4HB), which can be further converted to 4-aminobutyrate (GABA) or 1,4-butanediol (1,4-BDO) (Yim et al., 2011). GABA is used in the pharmaceutical and nutraceutical industry (Abdou et al., 2006), while 4HB and 1,4-BDO have applications as solvents or as components in polymers for medical applications (Martin and Williams, 2003; Saito et al., 1996) and compostable bioplastics (Chen et al., 2015). Microbial production of 4HB and 1,4-BDO currently uses *Escherichia coli* as a host, and titers of approximately 85 mM (4HB) and 200 mM (1,4-BDO) have been reported in micro-aerobic fed-batch fermentations (Yim et al., 2011). Neither the production of 4HB nor the production of 1,4-BDO via reduction of succinyl-CoA to succinate semialdehyde have hitherto been reported in *S. cerevisiae*. Exploration of yeast-based production of these commercially relevant compounds could benefit from the availability of a pathway that generates succinyl-CoA in the yeast cytosol.

Succinyl-CoA is produced by oxidative decarboxylation of α -ketoglutarate, mediated by the α -ketoglutarate dehydrogenase (α KGDH) complex. In eukaryotes, the α KGDH complex is present in the mitochondria, where it participates in the tricarboxylic acid cycle. The *S. cerevisiae* α KGDH complex is composed of three catalytic subunits: α -ketoglutarate dehydrogenase (E1, EC 1.2.4.2), dihydrolipoyl transsuccinylase (E2, EC 2.3.1.61) and dihydrolipoyl dehydrogenase (E3, EC 1.8.1.4) (Dickinson et al., 1986; Repetto and Tzagoloff, 1989, 1990). The structural subunit E4 was identified more recently in *S. cerevisiae* (Heublein et al., 2014) and has a role in recruiting the E3 subunit to the E1-E2 core. In *S. cerevisiae*, E1, E2 and E4 are specific for the α KGDH complex, whereas E3 is shared with the pyruvate dehydrogenase complex (Pettit and Reed, 1967).

Thiamine pyrophosphate (TPP) and lipoic acid are essential cofactors for activity of the α KGDH complex. TPP is involved in the decarboxylation reaction whereas lipoic acid is covalently bound to E2 and shuttles the substrate through the different catalytic domains (Bunik, 2003; Graham and Perham, 1990). Functional expression of an α KGDH complex in the yeast cytosol will therefore require availability of both TPP and lipoic acid in this cellular compartment. TPP is present in the yeast cytosol, where it acts as cofactor for transketolase and pyruvate decarboxylase (Hohmann and Meacock, 1998). Lipoic acid is, however, only present in yeast mitochondria, where it is synthesized from octanoyl-acyl-carrier-protein (octanoyl-ACP), an intermediate of fatty acid biosynthesis (Schonauer et al., 2009). Therefore, functional expression of an α KGDH complex in the yeast cytosol is expected to require the additional introduction of cytosolic enzymes required for its lipoylation (Kozak et al., 2014).

Lipoic acid biosynthesis and attachment are best understood in *E. coli*, which harbors a *de novo* biosynthetic pathway and a scavenging pathway (Spalding and Prigge, 2010). The *de novo* synthesis pathway uses octanoyl-ACP as substrate and involves two dedicated enzymes: Lipoyl(octanoyl)-ACP:protein transferase (LipB) and lipoyl synthase (LipA), of which the yeast homologs (Lip5 and Lip2, respectively) are located in the mitochondria. The *E. coli* scavenging pathway depends on a lipoate-protein ligase (LplA), which activates lipoate to lipoyl-AMP and attaches it to the target protein (Morris et al., 1995). The scavenging pathway is absent in *S. cerevisiae*, even though *LIP3*, a homolog of *E. coli lplA*, is required for *de novo* mitochondrial synthesis of lipoic acid (Schonauer et al., 2009).

The goal of the present study was to explore the functional expression of a heterologous α KGDH complex in the cytosol of *S. cerevisiae*. To this end, structural genes encoding subunits of the *E. coli* α KGDH complex and the lipoic acid scavenging gene *lplA* were expressed in *S. cerevisiae*. Functional expression was tested by assaying α KGDH activity in mitochondrial and cytosolic fractions of cell extracts. Size-exclusion chromatography and mass-spectrometry were used to investigate assembly of the complex and its lipoylation status. Finally, to explore *in vivo* functionality, a 5-aminolevulinic acid (ALA) auxotrophic strain was

constructed whose growth in the absence of ALA depended on cytosolic activity of the heterologously expressed α KGDH complex.

2. Materials and methods

2.1. Strains and maintenance

The *S. cerevisiae* strains used in this study (Table 2) share the CEN.PK113-7D genetic background (Entian and Kötter, 2007). Stock cultures of *S. cerevisiae* were grown aerobically in 500 mL shake flasks containing 100 mL synthetic medium (SM) (Verduyn et al., 1992) or YP medium (10 g L⁻¹ Bacto yeast extract, 20 g L⁻¹ Bacto peptone, 20 g L⁻¹ D-glucose). When needed, cultures were supplied with D-lipoic acid (Sigma-Aldrich, St. Louis, USA) to a final concentration of 100 μ g L⁻¹ or with 5-aminolevulinic acid (Sigma-Aldrich) to a final concentration of 50 mg L⁻¹. Stock cultures of *E. coli* XL1-Blue Subcloning Grade Competent Cells (Agilent Genomics, Santa Clara, USA) were grown in LB medium (5 g L⁻¹ Bacto yeast extract, 10 g L⁻¹ Bacto tryptone, 10 g L⁻¹ NaCl) supplemented with 100 mg L⁻¹ ampicillin. Media were autoclaved at 121 °C for 20 min. Media supplements and antibiotics were filter sterilized and added to the media prior to use. For yeast strain storage, glycerol was added to grown cultures to a final concentration of 30% v/v and 1 mL aliquots were stored at -80 °C.

2.2. Molecular biology techniques

Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific, Waltham, USA) was used for PCR amplification for cloning purposes. The manufacturer's protocol was followed except for the use of a low primer concentration (0.2 μ M instead of 0.5 μ M). Diagnostic PCR was performed with DreamTaq PCR Master Mix (2X) (Thermo Fisher Scientific). Desalted (DST) oligonucleotide primers were used, except for primers binding to coding regions, which were PAGE purified (Sigma Aldrich). For yeast colony PCR, genomic DNA was isolated as described by Lööke et al. (2011). Commercial kits for DNA extraction and purification were used for small-scale DNA isolation (Sigma Aldrich), PCR cleanup (Sigma Aldrich), and gel extraction (Zymo Research, Irvine, USA). Restriction analysis of constructed plasmids was performed using FastDigest restriction enzymes (Thermo Scientific). Gibson assembly of linear DNA fragments was performed using NEB-Builder HiFi DNA Assembly Master Mix (New England Biolabs) in a total reaction volume of 5 μ L. Transformation of chemically competent *E. coli* XL1-Blue was performed according to the manufacturer's protocol.

2.3. Plasmid construction

The plasmids and oligonucleotide primers used in this study are listed in Table 1 and in the Supplementary Table 1, respectively. Protein sequences of *E. coli* α KGDH subunits E1 (*Ec_sucA*, accession number P0AFG3), E2 (*Ec_sucB*, P0AFG6) and E3 (*Ec_lpd*, accession number P0A9P0), lipoate protein ligase LplA (*Ec_lplA*, P32099), and of *R. sphaeroides* 5-aminolevulinic acid synthase (*Rs_hemA*, Q04512), were used to order codon-optimized sequences from GeneArt (Regensburg, Germany). Codon usage was optimized with the GeneArt algorithm (Raab et al., 2010). Genes *Ec_sucA* and *Ec_sucB* were ordered as linear DNA fragments (GeneStrings, GeneArt), other genes were ordered in plasmids.

Plasmid pUD614 was constructed by Gibson assembly of the *Ec_sucA* ORF amplified from ordered linear DNA fragments using primers 10540 and 10541 and a vector backbone amplified from plasmid pUD301, using primers 6486 and 10562. Plasmid pUD616 was constructed by Gibson assembly of the *Ec_sucB* ORF amplified from the ordered linear DNA fragments using primers 10542 and 10543 and a vector backbone amplified from plasmid pUD302 using primers 3628 and 6494. Plasmid pUD618 was constructed by Gibson assembly of the *Ec_lplA* ORF amplified from pUD622 using primers 10544 and 10545 and a vector

Table 1
Plasmids used in this study.

Name	Relevant characteristic	Origin
pMEL11	2 μ m ori, <i>amdS</i> , pSNR52-gRNA.CAN1.Y-tSUP4	Mans et al. (2015)
pUDR119	<i>amdSYM</i> 2 μ m gRNA-SGA1	van Rossum et al. (2016a)
pUDR263	<i>amdSYM</i> 2 μ m gRNA-HEM1	This work
pUDR573	<i>amdSYM</i> 2 μ m gRNA-X-2	This work
pUD191	pMA-pTPI1- <i>Ec_lpd-tTEF1</i>	GeneArt, Germany
pUD482	pMA- <i>Rs_hemA</i>	GeneArt, Germany
pUD622	pMA- <i>Ec_lplA</i>	GeneArt, Germany
pUD301	pTPI1-pdhA-tTEF1	Kozak et al. (2014)
pUD302	pTDH3-pdhB-tCYC1	Kozak et al. (2014)
pUD303	pADH1-aceF-tPGI1	Kozak et al. (2014)
pUD304	pTEF1-lpd-tADH1	Kozak et al. (2014)
pUDE482	pTEF1-Venus-tENO2	Gorter de Vries et al. (2018)
pUD614	pTPI1- <i>Ec_sucA-tTEF1</i>	This work
pUD616	pTDH3- <i>Ec_sucB-tCYC1</i>	This work
pUD618	pADH1-lplA-tPGI1	This work
pUD623	pTEF1- <i>Rs_hemA-tENO2</i>	This work
pUD625	pTEF1- <i>IEc_lpd-tADH1</i>	This work

Table 2
Saccharomyces cerevisiae strains used in this study.

Strain name	Relevant genotype ^a	Origin
CEN.PK113-7D	Prototrophic reference, <i>MATa</i>	Entian and Kötter (2007)
IMX585	<i>MATa can1::cas9-natNT2</i>	Mans et al. (2015)
IMX1190	<i>MATa can1::cas9-natNT2 hem1Δ</i>	This study
IMX1230	<i>MATa can1::cas9-natNT2 hem1Δ; sga1Δ::Rs_hemA</i>	This study
IMX1401	<i>MATa can1::cas9-natNT2 hem1Δ; sga1Δ::Rs_hemA; X2::(αKGDH)</i>	This study

^a {αKGDH}: chromosomally integrated *E. coli* α-ketoglutarate dehydrogenase cluster, pTPI1-*Ec_sucA-tTEF1* pTDH3-*Ec_sucB-tCYC1* pTEF1-*Ec_lpd-tADH1* pADH1-lplA-tPGI1.

backbone amplified from plasmid pUD303 using primers 4671 and 6221. The *Ec_lpd* ORF was amplified from plasmid pUD191 using primers 11303 and 11304. The obtained PCR product was used as template for amplification with primers 11219 and 10561. Plasmid pUD625 was then constructed by Gibson assembly of the *Ec_lpd* ORF, obtained as specified above, and a vector backbone amplified from plasmid pUD304 using primers 3903 and 3904. Plasmid pUD623 was constructed via Gibson assembly of the *Rs_hemA* ORF amplified from pUD482 using primers 10560 and 10561 and a vector backbone amplified from plasmid pUDE482 using primers 3904 and 10563.

Plasmid pUDR263 was constructed by Gibson assembly of a double-stranded DNA fragment, obtained by annealing the complementary single-stranded oligonucleotides 10534 and 10535, and a vector backbone amplified from plasmid pMEL11 using primers 6005 and 6006. Plasmid pUDR573 was constructed by Gibson assembly of two linear fragments, both obtained via PCR amplification of plasmid pMEL11 using primer couples 5792–5980 and 5979–7374.

2.4. Strain construction

S. cerevisiae strains were transformed with the LiAc/ssDNA method (Gietz and Woods, 2002). The transformation mixture was plated on modified SM plates, in which (NH₄)₂SO₄ was replaced by 0.6 g L⁻¹ acetamide and 6.6 g L⁻¹ K₂SO₄ (SM-Ac). Single colonies were obtained by re-streaking three times on identical plates. Counter-selection for plasmid loss was performed on SM plates containing 2.3 g L⁻¹ fluor-oacetamide (SM-Fac) (Solis-Escalante et al., 2013). Gene deletions and integrations were performed as previously described (Mans et al., 2015). Expression cassettes were flanked by 60 bp short homology

repeats (SHRs) to allow assembly of the αKGDH cluster in the X-2 intergenic locus (Mikkelsen et al., 2012), and of the *Rs_hemA* cassette in the SGA1 locus by means of homologous recombination (Kuijpers et al., 2013).

Strain IMX1190 was constructed by transforming the Cas9 expressing strain IMX585 with plasmid pUD263 and a double stranded repair oligonucleotide obtained by annealing oligonucleotides 10536 and 10537. Strain IMX1230 was constructed by transforming strain IMX1190 with plasmid pUDR119 and a repair fragment obtained by PCR amplification of the *Rs_hemA* expression cassette from plasmid pUDE482 using primers 10710 and 10711.

Gene expression cassettes for integration of αKGDH genes were prepared as follows: the cassette containing the *Ec_sucA* ORF was amplified from plasmid pUD614 using primers 5654 and 8646; the cassette containing the *Ec_sucB* ORF was amplified from plasmid pUD616 using primers 3277 and 11186; the cassette containing the *Ec_lpd* ORF was amplified from plasmid pUD625 with primers 5652 and 5653; the cassette containing the *Ec_lplA* ORF was amplified from pUD618 using primers 3284 and 8645. The obtained fragments were co-transformed with pUDR573 into IMX1230, yielding strain IMX1401. After transformation, gRNA expression plasmids were removed by counter-selection as described above.

2.5. Media and cultivation

Shake-flask cultures were grown at 30 °C in 500 mL flasks containing 100 mL synthetic medium (Verduyn et al., 1992) with 20 g L⁻¹ glucose (SMD) in an Innova incubator shaker (New Brunswick Scientific, Edison, NJ, USA) set at 200 rpm. When required, media were supplemented with lipoic acid at a concentration of 100 μ g L⁻¹, or with 5-aminolevulinic acid at a concentration of 50 mg L⁻¹. Glucose-limited chemostat cultivation was performed at 30 °C in 2 L laboratory bioreactors (Applikon, Delft, The Netherlands) with a working volume of 1 L. For continuous cultures, synthetic medium was supplemented with 7.5 g L⁻¹ glucose (SMD) and 0.2 g L⁻¹ Pluronic PE6100 antifoam (BASF). For cultures supplemented with lipoic acid, a lipoic acid solution (50 g L⁻¹) in ethanol was prepared and added to the medium to a final concentration of 500 μ g L⁻¹. Continuous cultivation was preceded by a batch culture grown under the same conditions. When a rapid decrease in the CO₂ production indicated glucose depletion in the batch culture, continuous cultivation was initiated at a dilution rate of 0.10 h⁻¹. Culture pH was maintained at 5.0 by automatic addition of 2 M KOH. Bioreactors were sparged with 500 mL min⁻¹ air and stirred at 800 rpm to ensure fully aerobic conditions.

Sequential batch cultivation was carried out in bioreactors as indicated above, with the exception of the glucose concentration in the medium, which was increased to 20 g L⁻¹. Biomass was first grown in a bioreactor on synthetic medium supplemented with 5-aminolevulinic acid. Subsequently the biomass was harvested, washed twice to remove residual 5-aminolevulinic acid, and used to inoculate new reactors, containing SMD with or without lipoic acid supplementation.

2.6. Analytical methods

Culture optical density at 660 nm was measured with a Libra S11 spectrophotometer (Biochrom, Cambridge, United Kingdom). Metabolite concentrations in culture supernatants and media were analyzed using an Agilent 1260 Infinity HPLC system equipped with an Aminex HPX-87H ion exchange column, operated at 60 °C with 5 mM H₂SO₄ as mobile phase at a flow rate of 0.600 mL min⁻¹.

2.7. Separation of mitochondrial and cytosolic fractions

Separation of mitochondrial and cytosolic fractions of cell homogenates was performed as described previously (Lutik et al., 1998) with minor modifications. Zymolyase from *Arthobacter luteus* (20,000 U

g^{-1} , AMS Biotechnology Ltd., Abingdon, United Kingdom) was used. Biomass from CEN.PK113-7D and IMX1401 was harvested from glucose-limited chemostat cultures supplemented with $500 \mu g L^{-1}$ of lipoic acid. α KGDH activity was measured in the complete homogenate, as well as in the cytosolic and mitochondrial fractions. Protein concentrations of homogenates and fractions were determined using the Quick Start Bradford Protein Assay (Bio-Rad Laboratories Inc., Hercules, CA, USA) according to the supplier's manual, with bovine serum albumin (essentially fatty acid free, Sigma-Aldrich) as a standard.

2.8. α KGDH enzymatic activity measurements

α -Ketoglutarate dehydrogenase complex activity was tested in complete cell homogenates as well as in cytosolic and mitochondrial fractions. Prior to enzyme-activity assays, samples were dialyzed for 2 h at $4^\circ C$ using a 0.5 mL Slide-A-Lyzer dialysis cassette with a 10000 molecular-weight cut-off (Thermo Fisher Scientific). The samples were dialyzed in 100 mM potassium phosphate buffer (pH 7.5) at 500 \times sample volume. Activity of the α KGDH complex was measured at $30^\circ C$ in a Hitachi model U-3010 spectrophotometer (Sysmex, Norderstedt, Germany) by monitoring reduction of NAD^+ at 340 nm in a 1 mL reaction mixture containing 100 mM phosphate buffer (pH 8.0), 1 mM $MgCl_2$, 0.2 mM thiamine pyrophosphate, 2.5 mM NAD^+ , 5 mM α -ketoglutaric acid (disodium salt, dehydrate), 2 mM L-cysteine hydrochloride, 0.05% (v/v) Triton X-100, and 20–100 μL of cell extract. The reaction was started by addition of 0.15 mM Coenzyme A (trilithium salt). All reagents were purchased from Sigma Aldrich.

2.9. Purification of the α KGDH complex by size-exclusion chromatography

Separation of mitochondrial and cytosolic fractions was performed as described above. Cytosolic fractions were centrifuged ($4^\circ C$, 10 min at 47,000 $\times g$) and the supernatant was transferred to an Amicon Ultra-15 centrifugal unit (10 kDa cutoff; EMD Millipore Corporation, Billerica, MA) for protein concentration. In this step, the sorbitol-containing buffer (0.65 M sorbitol, 25 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, and 1 mM $MgCl_2$) used for cellular fractionation was replaced with chromatography buffer containing 100 mM potassium phosphate buffer (pH 7.0), 0.01% sodium azide, 5% glycerol, 0.1 mM ribosylthymine phosphate, and 0.1 mM dithiothreitol. The final volume was 10 mL. A 5 mL aliquot of the sample was applied to a HiPrep 16/60 Sephacryl S-500 HR size-exclusion chromatography column (GE Healthcare, Little Chalfont, Buckinghamshire, United Kingdom) mounted on a Bio-Rad chromatography system. Elution with chromatography buffer was performed at a flow rate of $0.5 mL min^{-1}$. Elution of proteins was followed using the embedded spectrophotometer set at 280 nm. Fractions of 2 mL each were collected and used to measure α KGDH activity. Protein concentrations of the fractions were determined with the Quick Start Bradford protein assay. Fractions obtained from the chromatographic column were stored at $-80^\circ C$.

2.10. Proteomic analysis

For the proteomic analysis of the size-exclusion chromatography fractions, sample preparation and HPLC-tandem mass spectrometry (MS/MS) were performed as described previously (Kozak et al., 2014; Lu et al., 2007). To analyze the post-translational modifications of the E2 subunit of the α KGDH, strain IMX1401 was grown on SMD supplemented with lipoic acid and 2.5 mg (wet weight) of biomass were harvested and quickly chilled on ice. Protein extraction was performed as previously described (Tong, 2011) with minor modifications. Prior to protein precipitation with trichloroacetic acid (Sigma Aldrich), cells were vortexed in 1.5 mL Eppendorf tubes containing 0.50 g glass beads (450–600 μm ; Sigma Aldrich). Three bursts of 30 s were performed and between bursts the sample was chilled on ice for at least 30 s. After washing the sample with acetone, it was dried and resuspended in 50 μL

of a 6 M urea, 200 mM ammonium bicarbonate (ABC) solution. The protein concentration was determined using a Nanodrop 2000 (Thermo Scientific) and normalized to $1 \mu g mL^{-1}$ in the same solution. An aliquot of 50 μg of protein was further processed by adding 15 μL of a 10 mM dithiothreitol solution in 200 mM ABC. After incubation for 1 h at $37^\circ C$, 30 μL of a 20 mM iodoacetamide (Sigma Aldrich) solution in 200 mM ABC was added and the sample was incubated in the dark for 30 min at room temperature. The protein solution was then diluted with ABC in order to reach a final urea concentration of 0.9 M. To digest the protein, 3 μL of the proteolytic enzyme GluC (Promega) was added to 150 μL of protein solution and digestion was performed overnight at $37^\circ C$.

The digested peptides were desalted using an Oasis HLB 96 well plate (Waters, Milford, USA) according to manufacturer protocol. The purified peptide eluate was further dried using a SpeedVac vacuum concentrator. The speed-vac dried peptide fraction was resuspended in an aqueous solution of 3% (v/v) acetonitrile and 0.1% (v/v) formic acid under careful vortexing. An aliquot corresponding to approximately 250 ng of protein digest was analyzed using a one-dimensional shot-gun proteomics approach as described by (Köchler et al., 2012) with minor modifications. Briefly, 1 μL of sample was analyzed using a nano-liquid-chromatography system consisting of an ESAY nano LC 1200, equipped with an Acclaim PepMap RSLC RP C18 separation column ($50 \mu m \times 150 mm$, $2 \mu m$, 100 \AA), and an QE plus Orbitrap mass spectrometer (Thermo Scientific). The flow rate was maintained at $300 nL min^{-1}$ over a linear gradient from 5% to 30% solvent B (in solvent A) over 90 min, and finally to 75% B over 25 min. Solvent A consisted of an aqueous solution containing 0.1% (v/v) formic acid, while solvent B consisted of 80% (v/v) acetonitrile in water and 0.1% (v/v) formic acid. The Orbitrap was operated in data dependent acquisition mode acquiring peptide signals from 400 to 1250 m/z at 70 K resolution, where the top 10 signals were isolated at a window of 2.0 m/z and fragmented using a NCE of 28. The AGC target was set to $5 \cdot 10^4$, at a max IT of 150 ms and 17.5 K resolution. In addition, Parallel Reaction Monitoring (PRM) was used to screen for the expected masses of both the unmodified and the lipoylated TDKVVLE peptide. For this fragmentation spectra were continuously acquired at 17.5 K resolution, $2 \cdot 10^5$ AGC target, max 100 msec IT, an isolation window of 2.0 Da and 28 NCE. To confirm complete lipoylation, a synthetic, unmodified peptide standard of sequence TDKVVLE was purchased from Genscript (Nanjing, China). A quantity of approximately 500 pg of peptide standard was injected in the LC-MS/MS system to determine retention time (Supplementary Fig. 4) and fragmentation profile. Database search and data processing. Raw data were analyzed using PEAKS Studio 8.5 (Bioinformatics Solutions Inc, Waterloo, Canada) allowing 20 ppm parent ion and 0.02 Da fragment mass error tolerance. Search conditions included considering 2 missed cleavages, carbamido methylation as fixed and K linked lipoyl (+188.03 Da), or carbamidomethylated lipoyl (+304.09 Da) groups as variable modifications. Data were analyzed against the *S. cerevisiae* protein database (Uniprot, June 2018, Tax ID 4932) where the protein sequence of dihydrolipoyllysine-residue succinyltransferase component of 2-oxoglutarate dehydrogenase complex (*sucB*, *E. coli*, POAFG6) was added manually. Database search included the GPM crap contaminant database (<https://www.thegpm.org/crap/>) and a decoy fusion for determining false discovery rates. Peptide spectrum matches were filtered against 1% false discovery rate (FDR) and protein identifications with 2 or more unique peptides were considered as significant hit. Data from PRM were analyzed manually for expected peptide hits.

3. Results

3.1. Expression of *E. coli* α -ketoglutarate dehydrogenase genes in *S. cerevisiae* leads to increased cytosolic α KGDH activity

To introduce a functional α -ketoglutarate dehydrogenase complex

(α KGDH) in the yeast cytosol, its three subunits need to be functionally expressed and assembled in this cellular compartment. Mitochondrial targeting of the subunits of the *S. cerevisiae* α KGDH complex is mediated by N-terminal amino-acid sequences (Dudek et al., 2013). Since prokaryotic enzymes are not expected to harbour sequences that target them to yeast mitochondria, the *E. coli* genes encoding the three α KGDH subunits (*Ec_sucA*, *Ec_sucB* and *Ec_lpd*) were selected for expression in *S. cerevisiae*. Activity of the α KGDH complex is dependent on lipoylation of the E2 subunit (Schonauer et al., 2009). Since the *S. cerevisiae* lipoylation system is confined to the mitochondrial matrix and therefore cannot lipoylate cytosolic proteins (Kozak et al., 2014), the *E. coli* gene encoding lipoyl-transferase (*Ec_lplA*; P32099) was also expressed. All four genes were expressed from constitutive promoters as a single $\{\alpha$ KGDH $\}$ cluster. To enable characterization of *in vivo* cytosolic α KGDH activity, the native mitochondrial 5-aminolevulinic acid (ALA) synthase gene *HEM1* was deleted and a cytosolic bacterial ALA synthase from *Rhodobacter sphaeroides* was integrated, resulting in *S. cerevisiae* strain IMX1401.

To investigate subcellular localization and activity of the heterologously expressed *E. coli* α KGDH, cells of strain IMX1401 (*hem1 Δ* ; *sga1 Δ ::Rs_hemA*; *X2::{\alpha*KGDH $\}$) and of the reference strain CEN.PK113-7D were harvested from steady-state aerobic, glucose-limited chemostat cultures supplemented with lipoic acid. Cell homogenates of these cultures were separated into cytosolic and mitochondrial fractions. For each fraction, α KGDH enzymatic activity was measured and the specific activity was calculated based on the protein content of the assayed fraction. α KGDH activity in homogenates of the reference strain *S. cerevisiae* CEN.PK113-7D was $0.005 \pm 0.002 \mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$. An over 10-fold higher activity ($0.056 \pm 0.015 \mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$), representing the combined activities of the native and heterologously expressed α KGDH complexes, was observed in the homogenate of strain IMX1401 (Fig. 1).

The activity of α KGDH in mitochondrial fractions of the homogenates of strains IMX1401 and CEN.PK113-7D showed a much smaller difference (0.122 ± 0.004 and $0.059 \pm 0.034 \mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$, respectively; Fig. 1). As anticipated based on the mitochondrial localization of the native *S. cerevisiae* α KGDH complex, only very low α KGDH activities were observed in cytosolic fractions of the reference strain CEN.PK113-7D ($0.002 \pm 0.001 \mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$). In contrast, activity in cytosolic fractions of strain IMX1401 was close to that of the total homogenates ($0.054 \pm 0.011 \mu\text{mol (mg protein)}^{-1} \text{min}^{-1}$). These results indicated that *S. cerevisiae* IMX1401 functionally expressed *E. coli* α KGDH in its cytosol.

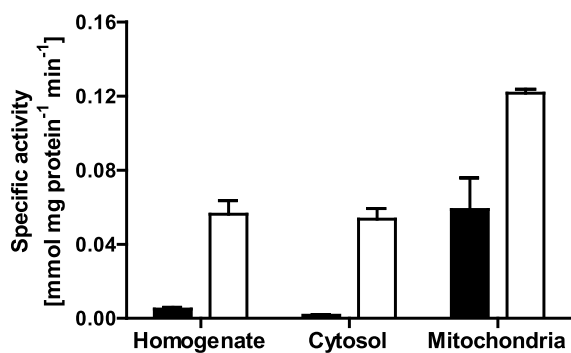


Fig. 1. α -Ketoglutarate dehydrogenase activity in complete homogenate, cytosolic and mitochondrial fractions of the reference strain *S. cerevisiae* CEN.PK113-7D (black bars) and strain IMX1401 (cytosolic α KGDH, *lplA*; white bars). The specific activity was calculated based on the protein content of the respective fraction. Yeast strains were pre-grown in aerobic, glucose-limited cultures supplemented with lipoic acid. Average and standard error were obtained from duplicate fractionation experiments for each strain.

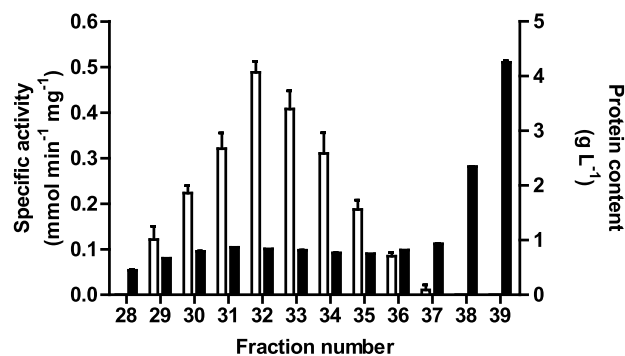


Fig. 2. α -Ketoglutarate dehydrogenase activity (white bars) and protein content (black bars) of the cytosolic fraction of a cell homogenate of *S. cerevisiae* IMX1401 (*hem1 Δ* , *sga1 Δ ::hemA*, *X2::{\alpha*KGDH $\}$) in fractions obtained by size-exclusion chromatography. Averages and standard errors were obtained from duplicate measurements performed for the same sample.

3.2. Size-exclusion chromatography and mass spectrometry reveal fully assembled and lipoylated α KGDH subunits in the yeast cytosol

Proteins in the cytosolic fraction of *S. cerevisiae* IMX1401 (*hem1 Δ* ; *sga1 Δ ::Rs_hemA*; *X2::{\alpha*KGDH $\}$) were separated by size-exclusion chromatography. α KGDH activity was first detected after 0.12 column volume changes (29 fractions of 2 mL, column volume of 482.5 mL, Fig. 2). Peak α KGDH activity was observed in fraction 32, and no activity was found beyond fraction 37. Fractions 29–31 and 32–34 were pooled (pools 1 and 2, respectively) and subjected to mass spectrometry to determine which native *S. cerevisiae* proteins co-eluted in the fractions with peak α KGDH activity and, thereby, to estimate the size of the heterologously expressed α KGDH complex. Peptides originating from each of the three structural *E. coli* α KGDH subunits were detected in pools 1 and 2, indicating that all three subunits were incorporated in the active complex. The main native *S. cerevisiae* proteins identified in the same fractions were ribosomal proteins (pool 1 and 2) and proteins of the proteasome (pool 2) (Supplementary Materials). These results indicate that the heterologous α KGDH complex is larger than the proteasome (2.4 MDa, as reported by Leggett et al. 2002) and at least as large as the ribosome (3.3 MDa, as reported by Ben-Shem et al., 2010).

To investigate the attachment of the lipoyl residue on the E2 subunit of the heterologously expressed α KGDH complex, proteins were extracted from an early exponential phase culture of strain IMX1401 grown aerobically on SMD supplemented with lipoic acid. Peptides generated by digestion with GluC, a proteolytic enzyme that cleaves at the C-terminus of either aspartic or glutamic acid residues (Drapeau et al., 1972), were analyzed by LC-MS/MS. Digestion yielded a peptide with the sequence TDKVVLE, which corresponded with the lipoylation target sequence of the *E. coli* E2 subunit. Mass and fragmentation profiles were coherent with a peptide of sequence TDKVVLE having a lipoic acid group attached at the target lysine (K) (Supplementary Fig. 3, panel B and C), while no unmodified peptide was found (Supplementary Fig. 3, panel A). The analytical capability to detect the unmodified peptide was confirmed by spiking a fully unmodified, synthetic peptide into the yeast lysate digest (Supplementary Fig. 4). The synthetic peptide was readily detected, confirming that all lysine residues on the heterologously expressed *E. coli* E2 subunit were indeed correctly lipoylated.

3.3. 5-Aminolevulinic acid auxotrophy of a *hem1 Δ* strain is complemented by cytosolic expression of *E. coli* α -ketoglutarate dehydrogenase and *Rhodobacter sphaeroides* aminolevulinic acid synthase

To test whether expression of *E. coli* α KGDH supports *in vivo*

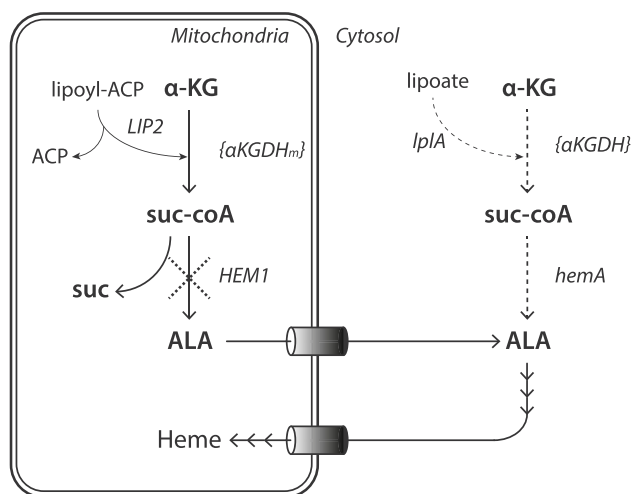


Fig. 3. Biosynthesis of 5-aminolevulinic acid (ALA) in *S. cerevisiae*. Solid lines represents the native pathway for ALA biosynthesis. Dashed lines indicate a heterologous ALA synthesis pathway, which is dependent on cytosolic α KGDH activity and able to complement deletion of *hem1*. α KG: α -ketoglutarate; suc-CoA: succinyl-CoA; suc: succinate; ALA: 5-aminolevulinic acid; ACP: acyl carrier protein; $\{\alpha$ KGDH $\}_m$: native α -ketoglutarate dehydrogenase enzyme complex; $\{\alpha$ KGDH $\}$: heterologous α -ketoglutarate dehydrogenase complex.

synthesis of succinyl-CoA in the yeast cytosol, its activity was connected to that of a cytosolically expressed heterologous enzyme that uses succinyl-CoA as a substrate. In *S. cerevisiae*, the heme precursor 5-aminolevulinic acid (ALA) is formed from succinyl-CoA and glycine in a condensation reaction catalyzed by the mitochondrial pyridoxal-5'-phosphate-dependent ALA-synthase Hem1 (Gollub et al., 1977; Moretti et al., 1993; Volland and Felix, 1984) (Fig. 3). Deletion of *HEM1* renders *S. cerevisiae* auxotrophic for ALA, which can be taken up from the medium by the GABA permease Uga4 (Garcia et al., 1997).

Deletion of *HEM1* in the Cas9-expressing *S. cerevisiae* reference strain IMX585 (Mans et al., 2015) yielded strain IMX1190. The specific growth rate of this *hem1* Δ strain on SMD supplemented with 50 mg L⁻¹ aminolevulinic acid was only slightly lower than that of the reference strain IMX585 (0.36 ± 0.00 h⁻¹ versus 0.38 ± 0.00 h⁻¹). Upon transfer to SMD without ALA supplementation, the *hem1* Δ strain continued to grow for approximately 5 further generations until growth arrest. This residual growth was attributed to carry-over of ALA or derived metabolites with the inoculum. Consistent with this notion, no growth was observed over a period of 200 h after transfer of biomass from this culture to a second culture on SMD without ALA. This observation confirmed the ALA auxotrophy of strain IMX1190 (Supplementary Fig. 1).

Integration of a heterologous, bacterial ALA synthase gene (*Rs_hemA*) from *Rhodobacter sphaeroides* in the genome of IMX1190 yielded strain IMX1230 (*hem1* Δ , *Rs_hemA*). Strain IMX1230 showed the same growth arrest upon ALA depletion as strain IMX1190 (Fig. 4). The observation that expression of *Rs_hemA* alone did not restore growth of a *hem1* Δ strain was attributed to the absence of succinyl-CoA, a substrate of the encoded ALA synthase, in the yeast cytosol.

Strain IMX1401 contained a deletion of the native mitochondrial ALA synthase gene *HEM1* and overexpression of a cytosolic bacterial ALA synthase. Therefore, ALA biosynthesis in IMX1401 was dependent on the cytosolic availability of the substrate succinyl-CoA. To investigate if *in vivo* cytosolic α KGDH activity could indeed support sufficient succinyl-CoA production to enable growth without ALA supplementation, strains IMX1401 (*hem1* Δ , *Rs_hemA*, $\{\alpha$ KGDH $\}$) and IMX1230 (*hem1* Δ , *Rs_hemA*) were grown in sequential bioreactor batch cultures (Fig. 5 and Supplementary Fig. 2, respectively). The first

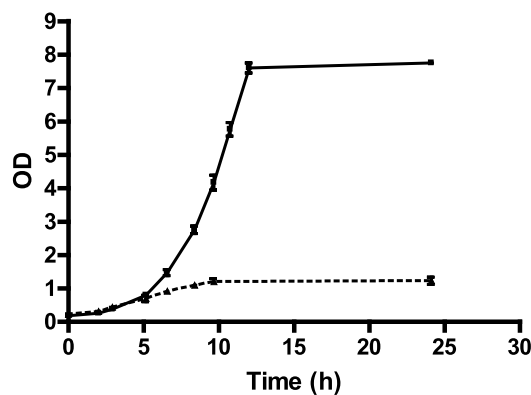


Fig. 4. Growth of *S. cerevisiae* IMX1230 (*hem1* Δ , *sga1* Δ ::*Rs_hemA*), lacking the native 5-aminolevulinic acid synthase (*Hem1*) and expressing a heterologous ALA synthase from *Rhodobacter sphaeroides* (*HemA*). Cells were pre-grown in SMD supplemented with ALA, harvested, washed three times and inoculated in SMD either in the presence (solid line) or in the absence (dotted line) of 5-aminolevulinic acid (ALA). Data are represented as average and standard error of measurements on duplicate growth experiments.

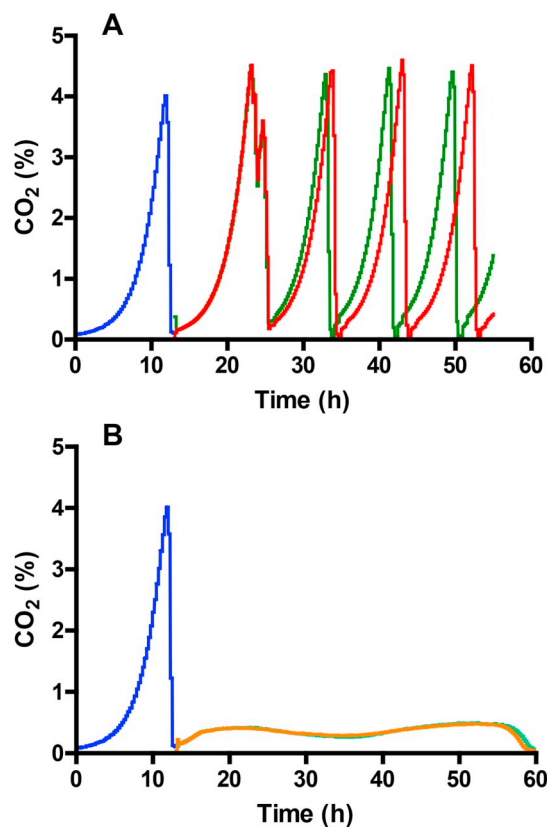


Fig. 5. Sequential bioreactor batch cultures of strain IMX1401 (*hem1* Δ , *sga1* Δ ::*Rs_hemA*, *X2*:: $\{\alpha$ KGDH $\}$). Growth was monitored by the CO₂ concentration in the exhaust gas of the cultures. Blue lines indicate CO₂ profiles of an initial batch culture on SMD supplemented with 50 mg L⁻¹ 5-aminolevulinic acid (ALA). Biomass grown in this culture was used as inoculum for subsequent bioreactor experiments without ALA supplementation. Panel A: cultivation on SMD supplemented with 100 µg L⁻¹ lipoic acid (red and green lines); Panel B: cultivation on SMD without supplementation (light green and orange lines). Except for the ALA-supplemented culture, duplicate cultures were run for each experimental condition. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

bioreactor batch culture was grown on SMD supplemented with ALA to allow for biomass formation. Upon glucose depletion, biomass was harvested, washed three times with sterile water to remove external ALA and used to inoculate duplicate bioreactor cultures on SMD with and without lipoic acid.

When strain IMX1401 (*hem1Δ, Rs_hemA, {αKGDH}*) was transferred to SMD without ALA and lipoic acid, no growth was observed over 40 h (Fig. 5, Panel B). In contrast, during four sequential batches of strain IMX1401 on SMD without ALA but with lipoic acid (Fig. 5, panel A), its specific growth rate ($0.37 \pm 0.00 \text{ h}^{-1}$, estimated from CO_2 production profiles) was almost identical to that of ALA-supplemented cultures (0.38 h^{-1}). The requirement of externally added lipoic acid for complementation of the ALA auxotrophy in strain IMX1401 (*hem1Δ, αKGDH, Rs_hemA*), combined with the inability of IMX1230 (*hem1Δ, Rs_hemA*) to grow in the presence of lipoic acid demonstrated *in vivo* αKGDH activity in the yeast cytosol, coupled to a heterologously expressed succinyl-CoA requiring enzyme.

4. Discussion

This study demonstrates the expression, assembly and *in vivo* activity of a bacterial α-ketoglutarate dehydrogenase complex in the cytosol of *S. cerevisiae*.

To test *in vivo* activity of the heterologous αKGDH complex, we used a strain in which synthesis of 5-aminolevulinic acid, the first committed precursor for heme biosynthesis, depended on cytosolic succinyl-CoA. Heme is required for functionality of the respiratory chain, the biosynthesis of sterols and as a cofactor for methionine biosynthesis. Since, especially during fermentative growth, only minute amounts of heme are required for these roles (Hanna et al., 2016), the tester strain was expected to be very sensitive to traces of cytosolic succinyl-CoA. The inability of strain IMX1230 (*hem1Δ; sga1Δ::Rs_hemA*) to grow without 5-aminolevulinic acid supplementation therefore confirmed that native supply cannot support efficient production of compounds that require cytosolic succinyl-CoA as a precursor. Although presence of cytosolic succinyl-CoA has not been described in *S. cerevisiae*, this compound has been proposed as succinyl donor for protein succinylation, which would require its availability outside of the mitochondria (Weinert et al., 2013; Zhang et al., 2010). The yeast strains described in this study provide an interesting platform for studies into the source of succinyl moieties for protein succinylation in *S. cerevisiae*.

Size-exclusion chromatography (Fig. 2) combined with mass spectrometry showed that the heterologously expressed αKGDH co-eluted with *S. cerevisiae* proteasome and ribosome subunits (Supplementary Materials). The *S. cerevisiae* proteasome and ribosome have reported masses of 2.4 MDa and 3.3 MDa, respectively (Leggett et al., 2002) (Ben-Shem et al., 2010). Our results are therefore in good agreement with the expected size of a fully assembled *E. coli* αKGDH complex, which has been described as 2.5–2.8 MDa according to Angelides and Hammes (1979) or 4.2 MDa based on the subunit stoichiometry described by Izard et al. (1999).

Expression of the *E. coli* αKGDH in the yeast cytosol provides a cytosolic source of succinyl-CoA, and thereby offer new possibilities for design and construction of yeast cell factories for industrially relevant compounds such as γ-aminobutyric acid, γ-hydroxybutyric acid, 5-aminolevulinic acid and 1,4-butanediol. Complementation of the ALA auxotrophy of an especially designed tester strain provides a proof of principle for succinyl-CoA synthesis in the yeast cytosol. The specific cytosolic αKGDH activity of the engineered strain IMX1401 was $0.056 \pm 0.015 \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$, while the activity of this enzyme in *E. coli* has been reported at $0.03 \pm 0.00 \mu\text{mol (mg protein)}^{-1} \text{ min}^{-1}$ (Li et al., 2006; Veit et al., 2007). Although the αKGDH activities between the native host and the engineered strain are comparable, further research is needed to assess and if necessary improve *in vivo* capacity of the heterologously expressed αKGDH complex to enable efficient production of succinyl-CoA derived chemicals.

Furthermore, while dependency on lipoate supplementation was useful for checking *in vivo* activity of the heterologously expressed αKGDH complex, industrial application can benefit from a published metabolic engineering strategy for lipoic acid synthesis in the cytosol of *S. cerevisiae* (Lian and Zhao, 2016).

Previously, ATP-independent production of cytosolic acetyl-CoA, which is crucial for yeast-based production of a wide range of industrially relevant compounds (de Kok et al., 2012; van Rossum et al., 2016b), has been achieved by cytosolic expression of bacterial pyruvate dehydrogenase complexes, whose size is similar to that of *E. coli* αKGDH (Kozak et al., 2014; Lian and Zhao, 2016). This concept was then used to couple acetyl-CoA synthesis via such a heterologously expressed pyruvate dehydrogenase complex to production of the polyketide tri-acetic acid lactone (Cardenas and Da Silva, 2016). Functional expression of a heterologous cytosolic αKGDH complex could thus represent the first step towards efficient production of succinyl-CoA derived products in yeast.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jymben.2019.10.001>.

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