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Engineering acetyl-CoA metabolic shortcut for eco-friendly production of polyketides triacetic acid lactone in *Yarrowia lipolytica*

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Abstract

Acetyl-CoA is the central metabolic node connecting glycolysis, Krebs cycle and fatty acids synthase. Plant-derived polyketides, are assembled from acetyl-CoA and malonyl-CoA, represent a large family of biological compounds with diversified bioactivity. Harnessing microbial bioconversion is considered as a feasible approach to large-scale production of polyketides from renewable feedstocks. Most of the current polyketide production platform relied on the lengthy glycolytic steps to provide acetyl-CoA, which inherently suffers from complex regulation with metabolically-costly cofactor/ATP requirements. Using the simplest polyketide triacetic acid lactone (TAL) as a target molecule, we demonstrate that acetate uptake pathway in oleaginous yeast (*Yarrowia lipolytica*) could function as an acetyl-CoA shortcut to achieve metabolic optimality in producing polyketides. We identified the metabolic bottlenecks to rewire acetate utilization for efficient TAL production in *Y. lipolytica*, including generation of the driving force for acetyl-CoA, malonyl-CoA and NADPH. The engineered strain, with the overexpression of endogenous acetyl-CoA carboxylase (ACC1), malic enzyme (MAE1) and a bacteria-derived cytosolic pyruvate dehydrogenase (PDH), affords robust TAL production with titer up to 4.76 g/L from industrial glacier acetic acid in shake flasks, representing 8.5-times improvement over the parental strain. The acetate-to-TAL conversion ratio (0.149 g/g) reaches 31.9% of the theoretical maximum yield. The carbon flux through this acetyl-CoA metabolic shortcut exceeds the carbon flux afforded by the native acetyl-CoA pathways. Potentially, acetic acid could be manufactured in large-quantity at low-cost from Syngas fermentation or heterogenous catalysis (methanol carbonylation). This alternative carbon sources present a metabolic advantage over glucose to unleash intrinsic pathway limitations and achieve high carbon conversion efficiency and cost-efficiency. This work also highlights that low-cost acetic acid could be sustainably upgraded to high-value polyketides by oleaginous yeast species in an eco-friendly and cost-efficient manner.

Keywords: *Yarrowia lipolytica*, acetic acid, metabolic shortcut, cytosolic acetyl-CoA, triacetic acid lactone (TAL), orthogonal pyruvate dehydrogenase complex

1. Introduction

To reduce our dependence on fossil fuels and mitigate climate change concerns, researchers have started seeking alternative routes to produce fuels and commodity chemicals. Motivated by the abundant supply of renewable feedstock and agricultural waste byproducts, biochemical engineers have been able to harness various cellular metabolism to build efficient microbe cell factories with the hope to upgrade the petroleum-based chemical manufacturing process. Our increasing knowledge on microbial physiology, biochemistry and cellular genetics and sophisticated synthetic biology tools have enabled us to explore a variety of host strains with the promise to produce large volume of green and commodity chemicals, including butanol, isobutanol, 1,3-PDO, 1,4-butanediol, organic acids, amino acids, and fatty acids-based fuels *et al* in the past two decades (Zambanini, Kleineberg et al. 2016, Liu, Hu et al. 2017, Liu, Zhao et al. 2017, Awasthi, Wang et al. 2018).

Triacetic acid lactone (TAL) is an important platform chemical with a broad spectrum of industrial applications ranging from organic synthesis, polymer plasticizers, adhesives to emulsifiers. It is the precursor for the synthesis of fungicides and fine chemicals including 1,3,5-trihydroxybenzene, resorcinol, phloroglucinol *et al* (Hansen 2002). TAL is currently manufactured from chemical catalysis with petroleum-derived dehydroacetic acid followed by acidolysis with concentrated sulfuric acid (H₂SO₄) at high temperature (Weissermel 2008). The use of petroleum feedstock, environmentally-unfriendly catalysts and the generation of toxic byproducts limit its industrial application as green chemicals (Chia, Schwartz et al. 2012). Therefore, there is a pressing need to develop low-cost and eco-friendly platform for sustainable production of TAL from renewable feedstocks.

By manipulating acetyl-CoA and malonyl-CoA metabolism in bacteria and yeast, metabolic engineers have established a number of TAL-producing host strains with *E. coli*, *S. cerevisiae*, *Y. lipolytica* *et al* (Tang, Qian et al. 2013, Cardenas and Da Silva 2014, Li, Qian et al. 2018, Markham, Palmer et al. 2018). Due to cellular toxicity issues, *E. coli*, was found to be a less promising producer to accumulate TAL (Xie, Shao et al. 2006). On the contrary, *S. cerevisiae* and *Y. lipolytica* were insensitive to TAL even at concentrations up to 200 mM (25.7 g/L), opening the opportunity for us to biologically synthesize TAL in large quantity. For example, a recent study has surveyed a large panel of metabolic pathways involving energy storage and generation, pentose biosynthesis, gluconeogenesis and lipid biosynthesis, to improve TAL production in *S. cerevisiae* (Cardenas and Da Silva 2014). In another study, recycling cytosolic NADPH and limiting intracellular acetyl-CoA transportation were also found as efficient strategies to enhance TAL accumulation in *S. cerevisiae* (Cardenas and Da Silva 2016). Compared with *S. cerevisiae*, *Y. lipolytica*, a natural

lipid producer, has recently been identified as an attractive industrial workhorse to produce various compounds due to its broad substrate range, high acetyl-CoA and malonyl-CoA flux as well as the availability of facile genetic manipulation tools (Markham and Alper 2018, Lv, Edwards et al. 2019). These features indicate that *Y. lipolytica* could be engineered as a superior host to produce acetyl-CoA and malonyl-CoA-derived compounds beyond lipid-based chemicals. Indeed, genetically rewired lipogenic pathways have achieved great success to produce lycopene, carotenoids, flavonoids and polyketides in *Y. lipolytica* (Xu, Li et al. 2014, Xu, Qiao et al. 2016, Qiao, Wasylenko et al. 2017, Xu, Qiao et al. 2017, Yu, Landberg et al. 2018). In a recent study, heterologous expression of 2-pyrone synthase along with the cytosolic expression of alternative acetyl-CoA pathways have been implemented to collectively improve TAL production up to 35.9 g/L in a bench-top reactors, with yield up to 43% of the theoretical yield from glucose (Markham, Palmer et al. 2018), highlighting the potential of using this yeast for large-scale production of polyketides.

Acetate presents in high levels in waste streams (municipal organic waste via anaerobic digestion) and lignocellulosic hydrolysates; acetate could also be synthesized from methanol carbonylation at very low cost (about \$340 per metric ton) (Qian, Zhang et al. 2016). And methanol could be abundantly produced by methane oxidation with heterogenous catalysis. These facts indicate that acetic acid will be a promising, sustainable and low-cost carbon source for industrial fermentation. To systematically understand the limiting cofactors associated with polyketide production in oleaginous yeast, we use TAL biosynthetic pathway as a testbed and investigated an arrange of biological factors including cofactor (NADPH) supply, orthogonal acetyl-CoA and malonyl-CoA availability, lipogenesis competition, substrate suitability and pH conditions (**Fig. 1**). Stepwise optimization of these parameters led to an 8.5-times improvement of TAL production (4.76 g/L) from industrial-grade acetic acid (glacial acid), with acetic acid-to-TAL yield at 31.9% of the theoretical yield. The reported acetyl-CoA shortcut pathway delineates a strong case that we could engineer *Y. lipolytica* as an eco-friendly platform to upgrade industrial acetic acids to commodity chemicals. This work also expands our capacity to produce green chemicals from sustainable feedstocks; in particular, acetic acid could be efficiently incorporated as carbon source for cost-efficient production of various polyketides in *Y. lipolytica*.

Fig. 1 Metabolic pathway for TAL synthesis in oleaginous yeast. MnDH, mannitol dehydrogenase; HXK, hexokinase; MAE1, malic enzyme; ACL2, ATP citrate lyase; IDP2, cytosolic NADP-specific isocitrate dehydrogenase; UGA2, succinate semialdehyde dehydrogenase; PYC1, pyruvate carboxylase; PDC1, pyruvate decarboxylase; ALD, aldehyde dehydrogenase; PDH, pyruvate dehydrogenase; ACS, acetic acid synthase; FAS1 and FAS2, fatty acid synthase; ACC1, acetyl-CoA-carboxylase; GH2PS, polyketide synthase.

2. Materials and methods

2.1 Strains and cultivation

Escherichia coli NEB5α high efficiency competent cells were purchased from NEB for plasmid construction, preparation, propagation and storage. The *Y. lipolytica* wild type strain W29 was purchased from ATCC (ATCC20460). The auxotrophic Polg (Leu-) was obtained from Eastern Biotech Company (Taipei, Taiwan)(Wong, Engel et al. 2017). All strains and plasmids used in this study are listed in supplementary Table S1.

LB broth or agar plate with 100 µg/mL ampicillin was used to cultivate *E. coli* strains. Yeast rich medium (YPD) was prepared with 20 g/ L Bacto peptone (Difco),

157 10 g/L yeast extract (Difco), and 20 g/L glucose (Sigma-Aldrich), and supplemented
158 with 15 g/L Bacto agar (Difco) for agar plates. YNB medium with carbon/nitrogen
159 ratio 10:1 for seed culture was made with 1.7 g/L yeast nitrogen base (without amino
160 acids and ammonium sulfate) (Difco), 5 g/L ammonium sulfate (Sigma-Aldrich), 0.69
161 g/L CSM-Leu (Sunrise Science Products, Inc.) and 20 g/L glucose. Selective YNB
162 plates were made with YNB media with carbon/nitrogen ratio 10:1 supplemented with
163 15 g/L Bacto agar (Difco). YNB fermentation medium with glucose as substrate and
164 carbon/nitrogen ratio 80:1 was made with 1.7 g/L yeast nitrogen base (without amino
165 acids and ammonium sulfate) (Difco), 1.1 g/L ammonium sulfate (Sigma-Aldrich),
166 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.) and 40 g/L glucose.

167 Phosphoric buffer solution (PBS) with pH 6.0 was made with 0.2 M Na_2HPO_4
168 and 0.2 M Na_2HPO_4 , which was used to replace water to make YNB- glucose-PBS
169 fermentation medium. YNB fermentation medium with sodium acetate as substrate
170 and carbon/nitrogen ratio 80:1 was made with 1.7 g/L yeast nitrogen base (without
171 amino acids and ammonium sulfate) (Difco), 0.825 g/L ammonium sulfate
172 (Sigma-Aldrich), 0.69 g/L CSM-Leu (Sunrise Science Products, Inc.), 41 g/L sodium
173 acetate. Glacial acetic acids were purchased from Sigma-Aldrich. Bromocresol purple
174 was a pH-sensitive indicator which could change its color with the pH from 5.2-7.0
175 (El-Ashgar, El-Basioni et al. 2012) and 40 mg/L bromocresol purple was added into
176 fermentation medium to indicate pH variation. The pH of medium was regulated to
177 6.0 by 6.0 M HCl in the fermentation process. And 1 mg/L cerulenin solution
178 prepared with dimethylsulfoxide (DMSO) was added into fermentation medium to
179 inhibit precursor competing pathway.

180 In TAL production process, single *Y. lipolytica* colonies were picked from YNB
181 selective plates and inoculated into YNB seed liquid media, which were grown at
182 30 °C for 48 h. For tube test, 100 μL seed cultures were inoculated into 5 mL
183 fermentation media in 50 mL tube. 600 μL seed cultures were inoculated into 30 mL
184 fermentation media in 250 mL shake flasks with 250 rpm and 30 °C. Time series
185 samples were taken for analyzing biomass, sugar content, and TAL titer.

186 2.2 Plasmid construction

187 All primers are listed in supplementary Table S2. All restriction enzymes were
188 purchased from Fisher FastDigest enzymes. Plasmid miniprep, PCR clean-up, and gel
189 DNA recoveries were using Zyppy and Zymoclean kits purchased from Zymo
190 research. All the genes, except for *GH2PS*, were PCR-amplified with the primers
191 from genomic DNA of *Y. lipolytica*, *S. cerevisiae*, *E. coli*, *B. subtilis*, *Aspergillus*
192 *nidulans*, respectively (Supplementary Table S1 and Table S2). *GH2PS* were
193 codon-optimized according to the human codon usage table. All these genes were
194 inserted into downstream of the *Y. lipolytica* TEF-intron promoter in the pYLXP'

vector backbone (Wong, Engel et al. 2017) at the SnaBI and KpnI site via Gibson assembly (Gibson, Young et al. 2009). Upon sequence verification by Genewiz, the restriction enzyme *AvrII*, *NheI*, *NotI*, *ClaI* and *SalI* (Fermentas, Thermo Fisher Scientific) were used to digest these vectors, and the donor DNA fragments were gel purified and assembled into the recipient vector containing previous pathway components via YaliBricks subcloning protocol (Wong, Holdridge et al. 2019). All assembled plasmids were verified by gel digestion and were subsequently transformed into the *Y. lipolytica* host strain Polg ΔLeu using the lithium acetate/single-strand carrier DNA/PEG method (Chen, Beckerich et al. 1997). In vector integration process, pYLXP' vector assembled with functional genes was linearized by restriction enzyme NotI (Fermentas, Thermo Fisher Scientific). The linear fragment was transformed into the *Y. lipolytica* host strain Polg ΔLeu for chromosomal gene integration.

2.3 Analytical methods

The cell growth was monitored by measuring the optical density at 600 nm (OD600) with a UV-vis spectrophotometer that could also be converted to dry cell weight (DCW) according to the calibration curve $DCW : OD600 = 0.33:1$ (g/L). The fermentation broth was centrifuged at 14,000 rpm for 5 min and the supernatant was used for analyzing the concentration of TAL, glucose, mannitol, acetic acid by HPLC with a Supelcogel™ Carbohydrate column (Sigma). Retention times for each tested chemical were listed in Supplementary Table S3. The quantification of TAL was analyzed by an UV absorbance detector at 280 nm, while glucose, mannitol, acetic acid was examined by a refractive index detector. The column was eluted with 10 mM H₂SO₄ at a column temperature of 50°C, a refractive index detector at temperature of 50°C, and a flow rate of 0.4 mL/min. All chemical standards were purchased from Sigma-Aldrich and standard curve were built to derive the area-concentration relationship. All reported results are three measures with standard deviations.

3. Results and discussion

3.1 Boosting precursor malonyl-CoA to improve TAL production in *Y. lipolytica*

It has been well characterized that the plant-derived 2-pyrose synthase (2-PS) is a type III polyketide synthase (Jez, Austin et al. 2000). Specifically, 2-PS from daisy family plant *Gerbera hybrida* was found to efficiently synthesize triacetic acid lactone (TAL) by joining one acetyl-CoA with two extender malonyl-CoA molecules (Jez, Ferrer et al. 2001, Austin and Noel 2003, Xie, Shao et al. 2006). To test TAL production, the coding sequence of *G. hybrida* 2-pyrone synthase (GH2PS, Uniprot ID: P48391) was codon-optimized and overexpressed in *Y. lipolytica*, yielding 0.5 g/L of TAL at 120 h with chemically-defined complete synthetic media (CSM-leu) in test tube (**Fig. 2A**), demonstrating the potential of using *Y. lipolytica* as a platform to

233 synthesize various polyketides.

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236 **Fig. 2** Engineering precursor pathway to improve TAL production in *Y. lipolytica*. (A)
 237 Comparison of TAL production from native acetyl-CoA carboxylase (ylACC1) and
 238 bacteria-derived acetyl-CoA carboxylase (EcACC). (B) Comparison of TAL
 239 production from alternative cytosolic NADPH pathways in *Y. lipolytica*.

240

241 It's generally believed that malonyl-CoA is the rate-limiting precursor for
 242 polyketide synthase (Zha, Rubin-Pitel et al. 2009, Xu, Ranganathan et al. 2011). We
 243 next sought to overexpress the bacteria-derived acetyl-CoA-carboxylase and the
 244 endogenous ACC1, to test whether the conversion of acetyl-CoA to malonyl-CoA
 245 would facilitate TAL synthesis. *E. coli* intracellular malonyl-CoA could be enhanced
 246 by overexpression of the four subunits of acetyl-CoA-carboxylase (accABCD) along
 247 with the expression of the biotinylating enzyme (biotin ligase encoded by *BirA*),
 248 leading to significantly improved production of both flavonoids and fatty acids
 249 (Leonard, Lim et al. 2007, Zha, Rubin-Pitel et al. 2009, Xu, Ranganathan et al. 2011,
 250 Xu, Gu et al. 2013). With the YaliBrick gene assembly platform (Wong, Engel et al.
 251 2017), we assembled the four catalyzing subunits (accABCD) and the biotinylating
 252 subunit (*BirA*). Interestingly, a negative effect was observed when these gene clusters
 253 were introduced to *Y. lipolytica*, resulting in 40% of TAL production compared to the
 254 parental strain. These results indicate that the bacterial-derived acetyl-CoA
 255 carboxylase could not be directly translated to yeast system, presumably due to the
 256 depletion of biotins or the protein burden stress of heterologous gene expression,
 257 albeit the specific TAL production (mg/gDCW) is about 4.2% higher (Fig. 2A).
 258 Endogenous *ylACC1*, a single polypeptide encoded by YALI0C11407 with two

internal introns removed, was also selected to co-express with *Gh2PS*, leading to TAL production increased to 0.66 g/L (Fig. 2A), indicating that overexpression of native ACC1 was beneficial for the TAL production. To test whether endogenous biotin ligase (BPL) was required to improve ACC1 activity (Pendini, Bailey et al. 2008), we co-expressed BPL1 (encoded by YALI0E3059). Similarly, degenerated production titer was observed in our experiment (Fig. 2A), indicating that biotin ligase is likely not the rate-limiting step for ACC1 activity in *Y. lipolytica*.

3.2 Recycling cytosolic NADPH to improve TAL production in *Y. lipolytica*

NADPH, the primary biological reducing equivalent to protect cell from oxidative stress and extend carbon-carbon backbones, has been reported as the major rate-limiting precursor in fatty acids and lipids synthesis in oleaginous species (Ratledge and Wynn 2002, Ratledge 2014, Wasylenko, Ahn et al. 2015, Qiao, Wasylenko et al. 2017). Source of cytosolic NADPH in Baker's yeast was contributed from various alternative routes (Minard, Jennings et al. 1998, Grabowska and Chelstowska 2003, Minard and McAlister-Henn 2005), depending on the carbon source and genetic background of the yeast strain. With glucose as carbon sources, cytosolic NADPH relies on the pentose phosphate pathway. Using TAL as the target molecule, we tested a collection of auxiliary cytosolic NADPH pathways (Liu, Marsafari et al. 2019) and investigated whether these alternative pathways would improve TAL production and cellular fitness (Fig. 1). These cytosolic NADPH pathways include malic enzyme (ylMAE), mannitol dehydrogenase (ylMnDH1, ylMnDH2), 6-phosphogluconate dehydrogenase (ylGND2) and succinate semialdehyde dehydrogenase (ylUGA2), which were reported to contribute to NADPH metabolism in various fungal species (Suye and Yokoyama 1985, Minard, Jennings et al. 1998, Wynn, bin Abdul Hamid et al. 1999, Grabowska and Chelstowska 2003, Minard and McAlister-Henn 2005, Rodríguez-Frómata, Gutiérrez et al. 2013, Liu, Marsafari et al. 2019).

Among these chosen NADPH source pathway, malic enzyme (MAE, encoded by YALI0E18634) presented the best results to improve TAL production (Fig. 2B). MAE was a NADP⁺-dependent enzyme that oxidatively decarboxylates malate to pyruvate and provides NADPH for lipid biosynthesis in oleaginous species (Ratledge 2014). When ylMAE was overexpressed with *GH2PS* and *ylACC1* (strain *HLYali101*), it showed a 60% increase in TAL titer and the volumetric production was increased to 0.8 g/L with a specific yield of 75.6 mg/g DCW (Fig. 2B). It is generally believed that ylMAE was localized in mitochondria (Zhang, Zhang et al. 2013, Ratledge 2014), we performed bioinformatic studies with TargetP 1.1 (Nielsen, Engelbrecht et al. 1997, Emanuelsson, Nielsen et al. 2000) and predicted that the first 78 nucleotides of

ylMAE encodes a 26 AA leader peptide which is responsible for mitochondria targeting. We then overexpressed the truncated ylMAE (t78ylMAE) with the N-terminal MTS removed to compare how the variation of ylMAE may affect TAL synthesis. Contrary to our hypothesis, removal of the MTS signal of ylMAE exhibits adverse effect on both TAL production and cell growth (**Fig. 2B**). qPCR results confirmed that the ylMAE mRNA abundance was 6.8 times higher compared to the strain without ylMAE overexpression. This result indicates that the 78 nt MTS sequence is essential to the function of ylMAE. Deletion of the N-terminal 78 nt MTS sequence renders the cell with less MAE catalytic efficiency and leads to degenerated production phenotype.

Protein subcellular location in fungi has been reported to not just depend on the leader peptide sequence. For example, both *S. cerevisiae* acetyl-CoA synthase (ACS) and *Y. lipolytica* malate dehydrogenase (MDH) could move across the mitochondria or peroxisome membrane boundary to function as cytosolic enzymes, depending on the carbon source and environmental conditions (Chen, Siewers et al. 2012, Kabran, Rossignol et al. 2012, Krivoruchko, Zhang et al. 2015). In particular, one of the proven mechanisms is related with alternative splicing of mRNA transcripts in *Y. lipolytica*: where the N-terminal mRNA sequence of malate dehydrogenase could be spliced in different ways to facilitate the mature peptide targeting to distinct cellular compartment (Mekouar, Blanc-Lenfle et al. 2010, Kabran, Rossignol et al. 2012). This mechanism indicates the complicated regulation of malic enzyme in *Y. lipolytica*, highlighting that overcoming metabolite trafficking may have positive effect on TAL accumulation. Increased TAL production could also be linked to the sufficient supply of precursor malonyl-CoA, possibly due to the balanced expression of Gh2PS and ACC1, which presumably creates a metabolic sink and pushes the equilibrium of ylMAE to function in the forward direction.

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324 ***3.3 Engineering orthogonal pyruvate dehydrogenase complex to boost TAL*** 325 ***production in Y. lipolytica***

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327 **Fig. 3** Engineering cytosolic acetyl-CoA pathway to improve TAL production in *Y.*
 328 *lipolytica*. YIPDC1, pyruvate decarboxylase; yIALD4, aldehyde dehydrogenase;
 329 EcPDH, pyruvate dehydrogenase comple; EcLplA, lipoate-protein ligase A.

330 Acetyl-CoA, derived from the central carbon metabolism (glycolysis and Krebs
 331 cycle), was the starting molecule in TAL synthesis. Acetyl-CoA is also the central hub
 332 for lipid synthesis, peroxisomal lipid oxidation and amino acid degradation pathway
 333 (Chen, Siewers et al. 2012, Krivoruchko, Zhang et al. 2015). Engineering alternative
 334 pathways to enhance cytosolic acetyl-CoA have been proven as efficient strategy to
 335 improve fatty acids and oleochemical production in both Bakers' yeast and *Y.*
 336 *lipolytica* (Kozak, van Rossum et al. 2014, van Rossum, Kozak et al. 2016, Xu, Qiao
 337 et al. 2016). Here we explored two independent metabolic engineering strategies to
 338 improve the oxidation of pyruvate to cytosolic acetyl-CoA. First, we investigated the
 339 endogenous pyruvate decarboxylase (PDC), acetylaldehyde dehydrogenase (ALD)
 340 and acetyl-CoA synthase (ACS) route, which decarboxylates pyruvate to acetaldehyde
 341 by PDC, oxidizes acetaldehyde to acetate by ALD, ligates acetate with CoASH to
 342 form acetyl-CoA by ACS (Flores, Rodríguez et al. 2000). Previous results have
 343 demonstrated that we can increase lipid production when the PDC-ALD genes were
 344 overexpressed in *Y. lipolytica* (Xu, Qiao et al. 2016). Comparison of the results by
 345 combinatorially overexpressing PDCs and ALDs along with GH2PS and MAE, the
 346 best result was obtained from the strain *HLYali102*, yielding only 0.4 g/L of TAL (**Fig.**
 347 **3**). We speculated that the synthesis process was possibly limited by the supply of
 348 malonyl-CoA. Endogenous *yIACC1* gene was further assembled to the previous
 349 pathway, however, no significant TAL improvement was observed in shake flasks
 350 (**Fig. 3**). These results indicate the complicated regulation of acetyl-CoA and
 351 malonyl-CoA metabolism. In particular, Snf1-mediated AMP kinase may
 352 phosphorylate ACC1 to repress ACC1 activity (Shi, Chen et al. 2014), the

353 accumulated acetyl-CoA may lead to histone acetylation and global transcriptional
354 change (Zhang, Galdieri et al. 2013). It could also point out a negative feedback loop
355 of acetyl-CoA regulation: accumulated acetyl-CoA and NADH will undergo oxidative
356 phosphorylation to generate ATP in mitochondria, which instead leads to weaker
357 phosphorylating activity of SNF1 and elevated ACC1 activity that converts
358 acetyl-CoA to malonyl-CoA (Mihaylova and Shaw 2011, Hsu, Liu et al. 2015).

359 We next attempted a bacterial-derived pyruvate dehydrogenase (PDH) complex
360 pathway, which is orthogonal to *Y. lipolytica* metabolism. Pyruvate dehydrogenase
361 complex (PDH), as a ubiquitous enzyme found in all kingdoms of life, catalyzed the
362 oxidative decarboxylation of pyruvate to acetyl-CoA, forming the connection between
363 glycolysis, the tricarboxylic acid cycle (TCA) and lipid biosynthesis (Kozak, van
364 Rossum et al. 2014). As for all eukaryotic life including *Y. lipolytica*, PDH was
365 localized in the mitochondrial matrix to provide acetyl-CoA for operating Krebs cycle.
366 This mitochondrial acetyl-CoA could not be converted to TAL in the cytosol. To solve
367 this challenge, we investigated whether the expression of the *E. coli* PDH would
368 benefit TAL synthesis in *Y. lipolytica*. Specifically, we assembled the genes encoding
369 the three catalytic subunits of PDH with our YaliBrick vectors (Wong, Engel et al.
370 2017): including pyruvate dehydrogenase (AceE, E1), dihydrolipoyllysine-residue
371 acetyltransferase (AceF, E2), and lipoamide dehydrogenase (LpdA, E3). Nevertheless,
372 when these three genes were assembled together and overexpressed along with
373 GH2PS, only 0.46 g/L TAL was detected (**Fig. 3**). It has been discovered recently that
374 the functional expression of bacterial PDH in *S. cerevisiae* requires the co-expression
375 of lipoate-protein ligase A (Kozak, van Rossum et al. 2014, Kozak, van Rossum et al.
376 2014, Nielsen 2014), which plays a pivotal role in lipoylating E2 subunits of PDH.
377 Similar to Baker's yeast, the two annotated lipoyltransferase or lipoate ligase in *Y.*
378 *lipolytica* (YALI0C03762 and YALI0F27357 respectively) are exclusively localized
379 in mitochondria (Sulo and Martin 1993, Hermes and Cronan 2013, Cronan 2016). To
380 enable cytosolic lipoylation and complement the bacterial PDH function, we next
381 co-expressed the lipoate-protein ligase A (LplA), TAL production was increased to
382 0.67 g/L with specific yield up to 87.72 mg/gDCW (strain HLYali103) (**Fig. 3** and
383 Table S4), indicating the essential role of EcLplA as an indispensable component for
384 the *E. coli* PDH functionality. This was the first report to functionally express the
385 bacteria-derived EcPDH with EcLplA in *Y. lipolytica*. Creating this orthogonal
386 acetyl-CoA route may bypass internal pathway regulations, providing a promising
387 path to overcome metabolic limitations and improve metabolite production in *Y.*
388 *lipolytica*.

389 Posttranslational modification of apoproteins with lipoyl groups was an
390 important process for many enzymes. There were two complementary systems for

protein lipoylation in *E. coli*: one was exogenous route using an ATP-dependent process by lipoate-protein ligase A (LplA); another one required lipoyl-[acyl-carrier protein]-protein-N-lipoyltransferase (LipB) to transfer endogenously synthesized lipoate to apoproteins (Miller, Busby et al. 2000). Both of these two proteins were localized in mitochondria, which presents a critical challenge to functionally express cytosolic PHDs in yeast. The current studies provide a viable option to functionally express bacteria-derived cytosolic PDH steps and leads to significantly improved TAL production. This PDH should be easily transferrable to other metabolite production systems, should there is a need to boost cytosolic acetyl-CoAs.

3.4 Comparison of TAL production from glucose and acetic acid

Fig. 4 Eco-friendly production of TAL from glucose and acetic acid. (A~C) Color variation of medium with pH indicator bromocresol purple. (A): Initial sodium acetate (NaAc) medium with pH 6.0; (B): Strain cultured in NaAc medium after 48 h with pH above 8.0; (C): Strain cultured in glucose medium after 48 h with pH below 4.0. (D): TAL production from minimal media supplemented with glucose by *HLYali101*; (E): TAL production from minimal media supplemented with NaAc by *HLYali101*. When NaAc was used as substrate, the medium pH will increase with the consumption of acetate. We adjusted the pH to 6.0 by adding HCl. Bromocresol purple is a

pH-sensitive indicator to track the pH variations of fermentation process.

A unique feature of *Y. lipolytica* is the high acetyl-CoA flux and its broad substrate range, which makes this yeast an attractive host to degrade organic waste (volatile fatty acids, alkanes *et al*). Previous metabolic engineering effort has demonstrated that *Y. lipolytica* possesses strong acetate utilization pathway that is equivalent or even superior to the hexose utilization pathway. For example, triacylglyceride (TAG) production could be improved from ~100 g/L to ~115 g/L in bench-top bioreactors, when the engineered strain was switched from glucose media (Qiao, Wasylenko et al. 2017) to acetate media (Xu, Liu et al. 2017). To investigate how different carbon sources may affect TAL synthesis, both glucose and sodium acetate were tested to compare TAL production efficiency in our engineered strains.

As acetic acid (HAc) has a relatively low pKa (4.75), we used 41 g/L sodium acetate (NaAc), equivalently to 29.5 g/L acetic acid (HAc) to cultivate the engineered strain. As the cell uptakes acetic acid, the cultivation pH will keep increasing. An *in situ* pH indicator (bromocresol purple) was used to track the pH change and 6 M HCl was used to adjust the pH in the shake flask (**Fig. 4A, 4B and 4C**). Controlling pH (**Fig. 4A**) led to the strain HLYali101 produced 1.05 g/L TAL in glucose-YNB medium, with 98% of glucose depleted and 11.53 g/L biomass produced (**Fig. 4D**). We also detected mannitol as a main byproduct, which increased to 3.7 g/L at 96 h and subsequently was utilized to support cell growth or TAL production (**Fig. 4D**). Strain HLYali101 produced about 1.29 g/L TAL from acetic acid with yield 300.69 mg/g DCW (**Fig. 4E**), this yield represents 230% improvement compared to the yield obtained from glucose medium (91.06 mg/g DCW) (**Fig. 4D and Table S4**). Depletion of acetic acid occurred at 144 h and the DCW reached 4.29 g/L, which is about half of the biomass from glucose.

Our data demonstrate that *Y. lipolytica* could efficiently uptake acetic acid as sole carbon source to produce polyketides. By harnessing the endogenous acetate uptake pathway, this externally provided acetic acid bypassed the pyruvate dehydrogenase steps and created a metabolic “shortcut” to acetyl-CoA with improved carbon conversion efficiency and pathway yield. This acetyl-CoA flux exceeded the capacity of the orthogonal PDH pathway when glucose is used as carbon source.

3.5 pH control and modulating precursor competing pathway to improve TAL production

Fig. 5 Improving TAL production by pH control and modulating precursor competing pathway in glucose-minimal media. Fermentation profile of glucose consumption, mannitol, dry cell weight, citric acid and TAL accumulation for strain *HLYali101* cultivated in glucose-minimal media conditioned with PBS buffer (A) or supplemented with 1 mg/L cerulenin (C). Fermentation profile of glucose consumption, mannitol, dry cell weight, citric acid and TAL accumulation for strain *HLYali103* cultivated in glucose-minimal media conditioned with PBS buffer (B) or supplemented with 1 mg/L cerulenin (D).

Despite that glucose is a preferred carbon source for cell growth, we constantly detected moderate or low TAL production in our engineered strains. The high TAL specific yield (300.69 mg/gDCW, Fig. 4E and Table S4) was only obtained in acetate media with pH control. We speculated that the low glucose-to-TAL yield was related with the pH declining of strain *HLYali101* (Fig. 4D): the pH of the fermentation media quickly decreased from 6 to 3.5 due to the accumulation of citric acid when glucose was utilized. This significant pH variation may negatively affect strain physiology, including cell membrane permeability and nutrients transportation process due to the loss of proton driving force. This eventually led to the accumulation of byproduct (mannitol and citric acid *et al*) and suboptimal TAL yield.

To compare how pH control may affect TAL production with the engineered

strain fermenting on glucose, we supplemented the minimal YNB media with 0.2 M phosphoric buffer solution (PBS, pH 6.0). Indeed, TAL production was increased to 2.04 g/L at 168 h, which was an increase of 60.1% and 94.3% respectively, compared with the results from pH controlled (supplementary Fig. S1) and uncontrolled (Fig. 4D) process. The major byproduct mannitol reached 4.2 g/L at 72 hour and citric acid reached 6.14 g/L at 96 h, and both were subsequently utilized (**Fig. 5A**). The biomass of strain *HLYali101* reached 8.66 g/L DCW with the TAL specific yield at 235.6 mg/gDCW (**Fig. 5A** and Table S4), which is still 27.6% lower than the specific yield from acetic acid (**Fig. 4E**). The glucose-to-TAL yield was 91.23 mg/g, which was 3.4 times higher than that of the pH uncontrolled fermentation process (**Fig. 4D** and Table S4). Strain *HLYali103* (orthogonal PDH expression) led to similar fermentation profile (**Fig. 5B**) when the glucose YNB media was conditioned with 0.2 M PBS buffer (pH 6.0). This strain led to the accumulation of 7.01 g/L biomass with the glucose-to-TAL yield at 98.44 mg/g glucose, about 7.9% higher than the yield obtained from strain *HLYali101*. Similarly, only ~50% of glucose was utilized in both strain *HLYali101* and *HLYali103* (**Fig. 5A** and **5B**), indicating the supplementation of PO_4^{3-} buffer may negatively impact the glucose uptake rate.

Y. lipolytica is a natural lipid producer capable of accumulating 30%~60% cell weight as lipid (Xu, Qiao et al. 2017). The internal fatty acid synthase (FAS) pathway strongly competes with TAL pathway for both acetyl-CoA and malonyl-CoA. In order to mitigate FAS competition, we attempted to use FAS inhibitor (cerulenin) to suppress lipid synthesis in our engineered strain. Cerulenin could irreversibly form a covalent adduct with the active site (cysteine residue) of β -ketoacyl-ACP synthase, inhibiting the incorporation of malonyl-CoA to the extending fatty acid backbone (Vance, Goldberg et al. 1972, Price, Choi et al. 2001). When 1 mg/L cerulenin was supplemented at 48 h, 3.2 g/L TAL was produced by strain *HLYali101* at 168 h (**Fig. 5C**), a 57% increase compared with the result without cerulenin (**Fig. 5A**). Byproducts mannitol and citric acid was decreased to 0.67 g/L and 3.75 g/L at 168 h, respectively. Glucose was depleted relatively faster compared to the strain without cerulenin (**Fig. 5A**). And the specific TAL production reached to 363.6 mg/gDCW, a 54% increase compared to the strain without cerulenin (**Fig. 5A** and Table S4). Similarly, strain *HLYali103* produced about 2.57 g/L TAL (**Fig. 5D**), a 32% increase compared to the strain without cerulenin (**Fig. 5B**). These results indicated that FAS pathway was the major competing pathway and malonyl-CoA flux became the main driver of TAL synthesis in the engineered strain *HLYali101* and *HLYali103*. Taken together, pH control and inhibition of FAS activity were effective strategies for diverting malonyl-CoA flux to TAL synthesis.

3.6 High-yield TAL production from acetic acid with chromosomally-integrated strains

Fig. 6. Test of TAL production with chromosomally-integrated pathway. Fermentation profile of NaAc consumption, mannitol, dry cell weight and TAL accumulation in strains LHYali105 (A) and LHYali106 (B).

The low biomass (Fig. 5C and 5D) and significant cost of cerulenin (10 mg for \$272 from Sigma Aldrich) prohibits the supplementation of cerulenin as chemical inhibitors to modulate malonyl-CoA flux in the engineered strain. On the other hand, ACC1, the key metabolic node of malonyl-CoA source pathway, was primarily controlled through the phosphorylation of serine residues by Snf1-mediated AMP kinase. The overflow of glucose metabolism or nitrogen starvation activates Snf1 kinase and slows down ACC1 activity (Hedbacker and Carlson 2008, Seip, Jackson et al. 2013, Zhang, Galdieri et al. 2013, Shi, Chen et al. 2014), which makes glucose an unfavorable carbon source for the production of malonyl-CoA-derived compounds. Since acetic acid could be directly converted to acetyl-CoA, we speculate that this acetyl-CoA shortcut may present little or no activation of the Snf1 kinase. In comparison with glucose, we hypothesize this alternative carbon source (acetic acid) will not cause inhibitory effect to the malonyl-CoA source pathway (ACC1).

To increase strain stability in acetic acid, we integrated the genes Gh2PS-ylMAE1-EcPDH-EcLplA and Gh2PS-ylMAE1-ylACC1-EcPDH-EcLplA (Fig. S2) into the chromosome of *Y. lipolytica*, leading to strains LHYali105 and LHYali106, respectively. The integrated strain LHYali105 yielded about 4.18 g/L TAL from YPA complex media (yeast extract, peptone and sodium acetate) in shake flask (Fig. 6A), a 37% increase compared to the strain LHYali105 grown in glucose-based YPD (yeast extract, peptone and dextrose) media (supplementary Fig. S3). With the

complementation of cytosolic PDH pathway, the strain LHYali106 yielded about 4.76 g/L TAL from YPA media (**Fig. 6B**), a 56% increase compared to the strain LHYali105 grown in YPD (supplementary Fig. S3). The biomass of LHYali106 reached 11.68 g/L at the end of fermentation with little mannitol (3.05 g/L) but no citric acid formation in the fermentation broth (**Fig. 6B**). The specific TAL yield reaches 407.5 mg/gDCW, which is 12.1% higher than the strain with cerulenin supplementation (**Fig. 5C**). The acetate-to-TAL conversion yield was increased to 0.149 g/g (Table S4), representing 31.9% of the theoretical maximum yield (0.467 g/g). This result demonstrates that acetic acid could lead to much higher TAL yield, exceeding the capability when glucose was used as carbon sources. Meanwhile, acetate has not been explored widely as sole carbon source in industrial fermentation. This work highlights the potential of engineering *Y. lipolytica* as a promising microbial platform for commercial manufacturing of high-value polyketides from low-cost acetic acids (glacial acetic acids).

4. Conclusion

In this work, triacetic acid lactone (TAL) biosynthetic pathway was constructed and optimized in oleaginous yeast *Y. lipolytica*. In addition to the codon-optimized 2-pyrone synthase gene, we found that both endogenous acetyl-CoA carboxylase (ylACC1) and malic enzyme (ylMAE1) are critical for the efficient conversion of acetyl-CoA and malonyl-CoA to TAL. Acetyl-CoA is the central hub of carbon metabolism, connecting with glycolysis, Krebs cycle and lipid synthesis. To unleash the potential of the heterologous polyketide synthase pathway, we assessed various cytosolic acetyl-CoA pathways to improve TAL synthesis. A bacteria-derived pyruvate dehydrogenase complex (Ec-PDH) along its cognate lipolyating protein (Ec-lplA) were identified as the most efficient cytosolic acetyl-CoA routes to improve TAL production. The engineered strain could efficiently ferment glucose or sodium acetate (NaAc) to TAL. In particular, the TAL titer (g/L) and specific yield (mgTAL/gDCW) from acetic acid media exceeds the titer and the specific yield obtained in glucose media. An *in situ* pH indicator (bromocresol purple) was used as a real-time pH reporter for process optimization in shake flasks. Suboptimal level of TAL accumulation (~3.2 g/L) was observed in a pH-controlled fermentation when the glucose media was supplemented with expensive FAS inhibitor (cerulenin). When the engineered strain was cultivated with acetate as sole carbon source in the absence of cerulenin, the TAL production was increased to 4.76 g/L in the chromosomally-integrated strain. The acetate-to-TAL yield reaches 0.149 g/g, representing 31.9% of the theoretical maximum yield (0.467 g/g). Since acetate uptake pathway essentially bypasses the mitochondrial PDH steps, the feeding of

acetate creates a metabolic shortcut to acetyl-CoA. The carbon flux through this acetyl-CoA metabolic shortcut exceeds the carbon flux afforded by the native acetyl-CoA pathways. This alternative carbon sources present a metabolic advantage over glucose, which may not elicit or activate Snf1-mediated AMP kinase repressing ACC1 activity. Potentially, acetic acid could be a readily-available, low-cost carbon sources from Syngas fermentation, anerobic digestion or heterogenous catalysis (methanol carbonylation).

This report demonstrates that *Y. lipolytica* possesses superior acetate uptake pathway to support both cell growth and metabolite production. Our engineering strategies highlights that acetic acid could be utilized as low-cost and sustainable carbon feedstock for efficient production of plant-derived polyketides in oleaginous yeast species. More importantly, the use of metabolic shortcut may overcome many intrinsic pathway limitations (kinetic or thermodynamic limitations), and facilitate high carbon conversion efficiency and volumetric titer. Since TAL is the simplest polyketide scaffold, the reported work may also be translated to other microbial platforms for upgrading waste acetic acids to high-value compounds in an eco-friendly and cost-efficient manner.

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Author contributions

PX conceived the topic. HL and MM performed genetic engineering and fermentation experiments. HL and PX wrote the manuscript.

Conflicts of interests

The authors declare that they have no competing interests. A provisional patent has been filed based on the results of this study.

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