Engineering cofactor and transport mechanisms in *Saccharomyces cerevisiae* for enhanced acetyl-CoA and polyketide biosynthesis

Javier Cardenas^a and Nancy A. Da Silva^{a*}

^a Department of Chemical Engineering and Materials Science, University of California, Irvine, CA, 92697-2575 USA

*Corresponding author

Tel: (+1) 949-824-8288

Fax: (+1) 949-824-2541

E-mail: ndasilva@uci.edu

Abstract

Synthesis of polyketides at high titer and yield is important for producing pharmaceuticals and biorenewable chemical precursors. In this work, we engineered cofactor and transport pathways in *Saccharomyces cerevisiae* to increase acetyl-CoA, an important polyketide building block. The highly regulated yeast pyruvate dehydrogenase bypass pathway was supplemented by overexpressing a modified *Escherichia coli* pyruvate dehydrogenase complex (PDHm) that accepts NADP⁺ for acetyl-CoA production. After 24 h of cultivation, a 3.7-fold increase in NADPH/NADP⁺ ratio was observed relative to the base strain, and a 2.2-fold increase relative to introduction of the native *E. coli* PDH. Both *E. coli* pathways increased acetyl-CoA levels approximately 2-fold relative to the yeast base strain. Combining PDHm with a *ZWF1* deletion to block the major yeast NADPH biosynthesis pathway resulted in a 12-fold NADPH boost and a 2.2-fold increase in acetyl-CoA. At 48 h, only this coupled approach showed increased acetyl-CoA levels, 3.0-fold higher than that of the base strain. The impact on polyketide synthesis was evaluated in a *S. cerevisiae* strain expressing the *Gerbera hybrida* 2-pyrone synthase (2-PS) for the production of the polyketide triacetic acid lactone (TAL). Titers of TAL relative to the base strain improved only 30% with the native *E. coli* PDH, but 3.0-fold with PDHm and 4.4-fold with PDHm in the $\Delta zwf1$ strain. Carbon was further routed toward TAL production by reducing mitochondrial transport of pyruvate and acetyl-CoA; deletions in genes *POR2*, *MPC2*, *PDA1*, or *YAT2* each increased titer 2 to 3-fold over the base strain (up to 0.8 g/L), and in combination to 1.4 g/L. Combining the two approaches (NADPH-generating acetyl-CoA pathway plus reduced metabolite flux into the mitochondria) resulted in a final TAL titer of 1.6 g/L, a 6-fold increase over the non-engineered yeast strain, and 35% of theoretical yield (0.16 g/g glucose), the highest reported to date. These biological driving forces present new avenues for improving high-yield production of acetyl-CoA derived compounds.

Key Words: *Saccharomyces cerevisiae*; pyruvate dehydrogenase; acetyl-CoA; mitochondrial pyruvate carrier; triacetic acid lactone

1. Introduction

Polyketides are a diverse group of natural products with valuable properties useful for the manufacture of biologics and pharmaceuticals as well as replacement of fossil-fuel derived materials (Jeandet et al., 2013; Li and Vederas, 2009; Nikolau et al., 2008). The synthesis of these molecules require polyketide synthase (PKS) systems that catalyze a complex set of reactions including decarboxylation, condensation, cyclization, and aromatization (Hertweck, 2009; Jez et al., 2002). Limitations in the native host include biosynthesis of small quantities and a lack of genetic tools for strain manipulations (Jeandet et al., 2012; Lussier et al., 2012; Santos et al., 2011); therefore, engineering of heterologous microbial cell factories is of great interest (Krivoruchko and Nielsen, 2015). The yeast *Saccharomyces cerevisiae* is advantageous as it is capable of synthesizing fungal and plant polyketides that are toxic to or difficult to produce in bacteria (Cardenas and Da Silva, 2014; Ma et al., 2009), and also allow the synthesis of complex polyketides relying on cytochrome P450 activities (Howat et al., 2014). Various types of PKSs are active in yeast, and the synthesis of fungal polyketides (6-methylsalicylic acid, dihydromonacolin L) as biologics (Kealey et al., 1998; Ma et al., 2009; Wattanachaisaereekul et al., 2007; Xu et al., 2013) and a plant polyketide (triacetic acid lactone, TAL) as a biorenewable chemical precursor (Cardenas and Da Silva, 2014; Saunders et al., 2015; Schwartz et al., 2014) has demonstrated the promise of yeast for polyketide biosynthesis.

Many polyketides are built from the simple building blocks acetyl-CoA and malonyl-CoA. These starter metabolites are in high demand in yeast, e.g., for fatty acid synthesis (Jong et al., 2014) and energy via ATP (Pronk et al., 1996). In order to synthesize polyketides at high levels, it is therefore necessary to improve availability of these starter metabolites and relevant cofactor pools. One of the most direct methods for improving synthesis of acetyl-CoA and

malonyl-CoA is by overexpressing the enzymes responsible for their formation and eliminating competing pathways. Using various overexpression and pathway disruption strategies, several studies have reported interventions aimed at increasing acetyl-CoA for the synthesis of sesquiterpenes (Chen et al., 2010, 2013; Shiba et al., 2007), butanol (Krivoruchko et al., 2013; Lian et al., 2014), polyhydroxybutyrate (PHB) (Kocharin et al., 2013, 2012), as well as fatty acids (Li et al., 2014; X. Tang et al., 2013). For malonyl-CoA formation, acetyl-CoA carboxylase (Acc1) is important and can limit malonyl-CoA synthesis. This can be circumvented by overexpression (Runguphan and Keasling, 2014; Wattanachaisaereekul et al., 2008) or prevention of deactivation of the Acc1 enzyme following glucose consumption (Choi and Da Silva, 2014; Hofbauer et al., 2014; Shi et al., 2014). In this recent work in our lab, the overexpression of a deactivation-resistant Acc1 yielded a 9-fold increase in activity *in vivo* leading to a 3-fold increase in both fatty acid and polyketide levels.

To engineer *S. cerevisiae* for increases in the starter metabolites acetyl-CoA and malonyl-CoA in the cytosol, it is also important to focus on the availability of pyruvate in the cell. Pyruvate is a major branch point for carbon following glycolysis (Pronk et al., 1996), one of which is the synthesis of acetyl-CoA via the PDH bypass (see Figure 1). Pyruvate is first decarboxylated; the Ald6 dehydrogenase then generates acetate that is converted to acetyl-CoA by the acetyl-CoA synthetase (Acs1/2). Recent computational optimizations rerouted carbon flux from the PDH bypass through an *E. coli* PDH to increase levels of the polyketide TAL; this also conserves ATP by eliminating use of the ATP-dependent Acs1/2 (Chowdhury et al., 2014). In this *in silico* study, the native PDH bypass was downregulated, and only use of an engineered NADP⁺-dependent *E. coli* PDH predicted an improved yield of TAL. Using the native *E. coli* PDH created a redox imbalance due to the NAD⁺-dependent nature of the enzyme, resulting in

no increase in TAL yield. A recent experimental approach in *S. cerevisiae* utilized various acetyl-CoA producing pathways including the *E. coli* PDH which increased isobutanol levels; the authors commented that a reduced energy input may have been a contributing factor (Lian et al., 2014). The use of cofactors to further increase carbon flux through engineered pathways has been demonstrated for the production of diverse products in yeast, including fatty acid ethyl esters (FAEEs), polyhydroxybutyrate (PHB), and 7-dehydrocholesterol (Jong et al., 2014; Kocharin et al., 2013; Su et al., 2015). These studies support altering host cofactor levels for improved product formation.

Pyruvate can also be utilized by other pathways after its carboxylation or transport to the mitochondria. The yeast mitochondrial pyruvate carrier (MPC) has been shown to transport pyruvate into the mitochondria (Bricker et al., 2012; Herzig et al., 2012). Similarly, acetyl-CoA can be transported using the carnitine shuttle system where carnitine acetyltransferases (CATs) are responsible for first priming acetyl-CoA for transport (via Yac2) before facilitating movement into the mitochondria (Yac1) where acetyl-CoA is regenerated (Cat2) (Franken et al., 2008). Pyruvate and acetyl-CoA are thus invaluable to the cell, and rely on these systems for transport into the mitochondria causing a significant drain on their cytosolic availability. Impeding this transport may result in larger cytosolic pools and thus increase polyketide synthesis in yeast.

The overall aim of our work was to engineer novel modifications to cofactor and transport pathways in *S. cerevisiae* to directly increase acetyl-CoA levels and thus polyketide synthesis. To increase acetyl-CoA, we expressed (for the first time) the NADP⁺ variant of the *E. coli* pyruvate dehydrogenase complex (PDHm) in yeast, and NADPH/NADP⁺ ratios and acetyl-CoA were compared to those for the base strain and one expressing the native *E. coli* PDH. PDHm overexpression was then coupled with a *ZWF1* gene deletion (to eliminate the major

yeast NADPH-producing pathway) to further drive carbon flux to acetyl-CoA, and the effects on the synthesis of the polyketide triacetic acid lactone (TAL) were determined. Additional pathway design incorporated key gene disruptions that reduced mitochondrial transport of pyruvate and acetyl-CoA. This novel strategy to increase cytosolic levels of pyruvate and acetyl-CoA in *S. cerevisiae* was assessed by evaluating the effects on TAL biosynthesis. The hindered transport and NADPH/acetyl-CoA upregulation strategies were then combined to further increase TAL production in the host. We demonstrate how increasing metabolite levels via these strategies can increase precursor levels and thus production of polyketides.

2. Materials and Methods

2.1 Strains and plasmids

Escherichia coli strain XL1-Blue (Stratagene, Santa Clara, CA) was used for plasmid amplification and *E. coli* NEB 10-beta cells (New England Biolabs, Ipswich, MA) were used for amplification of Gibson-assembled vectors. *Saccharomyces cerevisiae* strain BY4741 and various single gene deletion strains were obtained from Open Biosystems (Huntsville, AL). Further deletion strains were constructed using loxP-flanked *MET15*, *LEU2-d8*, *TRP1*, and *HIS3* selection markers amplified from our pXP vector library (Fang et al., 2011). The amplification primers contained approximately 50bp homology to locations flanking the target gene location. Yeast cells were transformed as described previously (Gietz et al., 1992; Hill et al., 1991) and gene disruptions were verified following amplification of genomic DNA using Taq polymerase (New England Biolabs, Ipswich, MA). Details of the strain construction and all primer sequences can be found in the Supplementary Data (Table S1). The complete list of *S. cerevisiae* strains is given in Table 1.

Plasmid pXP842-2PS harbors the *G. hybrida g2ps1* gene encoding 2-pyrone synthase (Cardenas and Da Silva, 2014). To express the *E. coli* PDH complex, a 2 μ -based trigenic vector was constructed. DNA fragments were created to flank the three PDH genes with promoters and terminators as listed: *ADH2p-lpd1-ADH2t*, *ADH1Mp-aceF-ADH2t*, and *HXT7p-aceE-CYCt*. The three genes were isolated by PCR from *E. coli* chromosomal DNA preparations (Dale and Greenaway, 1985). The *lpd1* and *aceF* fragments were placed in opposite orientation to prevent recombination of the terminator sequences. *ADH1Mp* refers to the medium truncated *ADH1* promoter which alters glucose-induced expression to approximately constitutive expression (Ruohonen et al., 1995). Template vector pXP843 (Leber and Da Silva, 2013) was linearized by BamHI / AatII (New England Biolabs, Ipswich, MA). Gibson assembly (Gibson et al., 2009) using the Phusion Hot Start II DNA Polymerase (New England Biolabs, Ipswich, MA) was performed using this linearized backbone and the three gene fragments listed above to generate pJCT-PDH. For construction of the alternate trigenic vector pJCT-PDHm for expression of the variant *E. coli* PDHm complex, the wildtype sequence for the *lpd1* gene was replaced with a variant incorporating 15 base changes using insertion mutagenesis (Lee et al., 2010). These modifications coincide with the amino acid changes previously identified for NADP⁺ binding (Bocanegra et al., 1993) as well as optimal codon usage preferences in *S. cerevisiae* (Table S2). Further details for construction of pJCT-PDH and pJCT-PDHm including all primers can be found in the Supplementary Data (Table S1, Figure S1). All plasmids used and constructed can be found in Table 1. Unless otherwise stated, all PCR-based amplifications were performed using KOD Hot Start DNA Polymerase (EMD Chemicals, San Diego, CA). Oligonucleotides used in this study were provided by IDT DNA (San Diego, CA), the GeneJetTM Plasmid Miniprep Kit (Thermo Scientific, Waltham, MA) was used for plasmid preparation, and DNA

sequences were confirmed for all PCR-amplified inserts (Eton Biosciences, San Diego, CA).

2.2 Media and cultivation

Luria-Bertani (LB) media was used for cultivation of XL1-Blue cells, with 150 mg/L ampicillin added for selection of plasmid-containing cells (Sambrook and Russell, 2001). Yeast selective SDC medium (2% dextrose, 0.5% casamino acids, 0.67% yeast nitrogen base, 0.5% ammonium sulfate supplemented with 100 mg/L adenine sulfate) and SD minimal medium (2% dextrose, 0.67% yeast nitrogen base, 0.5% ammonium sulfate and supplemented with 100 mg/L adenine sulfate, 100 mg/L L-tryptophan, 100 mg/L L-histidine-HCL, 100 mg/L L-methionine, and/or 150 mg/L L-leucine) were used for seed cultures. Complex YPD medium (1% dextrose, 1% yeast extract, 2% peptone) was used for the NADPH/NADP⁺ and acetyl-CoA quantification studies and for TAL expression. The *g2ps1*, *lpd1*, and *lpdlm* genes were expressed from the *S. cerevisiae* late-phase *ADH2* promoter that is active after glucose depletion and has highest expression levels in 1% glucose YPD medium (Lee and Da Silva, 2005). Due to the late expression, plasmid stabilities remain high even in this complex medium (Shen et al., 2012).

S. cerevisiae strains were grown overnight in 5 mL selective media in an air shaker (New Brunswick Scientific) at 250 rpm and 30°C, and used to inoculate 5 mL tube cultures to an initial cell density (OD₆₀₀) of 0.3 (Shimadzu UV-2450 UV-VIS Spectrophotometer, Columbia, MD). A correlation factor was used to convert OD measurements to dry cell weight per liter (Cardenas and Da Silva, 2014). For NADPH/NADP⁺ and acetyl-CoA quantification, samples were taken at 12, 24, and 48 h. For measuring polyketide TAL titers in the culture broth, the supernatant at 48 h was collected for HPLC analysis.

2.3 Plasmid stability

The yeast strains BYt and BYtB Δ zwf1 Δ por2 Δ mpc2 Δ pda1 Δ yat2 (Table 1) transformed with pXP842-2PS and pJCT-PDHm were cultivated in 1% YPD medium for 48 h, and plated onto YPD plates following serial dilutions with sterile water. After colonies were allowed to grow for 2-3 days, cells were replica plated to SDC plates supplemented with either 100 mg/L L-tryptophan or 100 mg/L uracil, as well as to non-selective YPD plates (to verify cell viability and transfer). The percentage of plasmid-containing cells was determined as the number of colonies on the selective plates divided by the number of viable colonies transferred. The plasmid stability was calculated for each individual plasmid as well as in combination. Three independent experiments were performed.

2.4 Quantitative NADPH / NADP⁺ assay

Both NADPH and NADP⁺ were quantified using a colorimetric 96-well plate assay (Sigma-Aldrich, St Louis, MO). Following a cold buffer (PBS) wash of the cells, a combination of 2 freeze / thaw cycles and homogenization was performed prior to deproteinization using a 10 kD cut-off spin filter (Sigma-Aldrich, St. Louis, MO). The cycling reaction was performed, and absorbance taken at 450 nm. A SpectraMax plate reader using SoftMax software was used for data acquisition (Molecular Devices, Sunnyvale, CA).

2.5 Acetyl-CoA assay

A boiling ethanol extraction was used on collected cell pellets for acetyl-CoA analysis, based on published procedures (Gonzalez et al., 1997; Villas-Bôas et al., 2005). Following release of the metabolites, nitrogen-supported solvent evaporation provided rapid sample concentration at room temperature. Following complete solvent evaporation, 200 μ L Sigma

acetyl-CoA assay buffer was added for resuspension. A fluorescence-based 96-well acetyl-CoA plate assay was used (Sigma-Aldrich, St. Louis, MO), and concentrated acetyl-CoA identified using wavelengths 535 nm (excitation) and 587 nm (emission) on a SpectraMax plate reader using SoftMax software for data acquisition.

2.6 Polyketide detection by HPLC

The concentration of triacetic acid lactone was measured by HPLC using the following Shimadzu configuration: LC-10AT pumps (Shimadzu), UV-VIS detector (SPD-10A VP, Shimadzu), and Zorbax SB-C18 reversed-phase column (2.1x150 mm, Agilent Technologies). Acetic acid-buffered (1%) acetonitrile and water were used as the mobile and aqueous phases, respectively. A gradient program using a 95-85% Pump B gradient (H₂O with 1% acetic acid) provided an elution time of approximately 12 minutes (flow rate 0.25 mL/min, column temperature 25°C).

3. Results and Discussion

3.1 Enhancement of NADPH/NADP⁺ and acetyl-CoA using PDH or PDHm overexpression

The synthesis of polyketides requires the availability of starter metabolites that react to form the final product. Triacetic acid lactone (TAL) is an acetyl-CoA derived polyketide that requires one acetyl-CoA and two malonyl-CoA units. Since cytosolic malonyl-CoA is formed directly from acetyl-CoA, the pool of acetyl-CoA was increased through a non-native pathway. The *E. coli* pyruvate dehydrogenase complex (PDH), responsible for converting pyruvate to acetyl-CoA, was introduced to supplement the highly-regulated and ATP-requiring yeast pathway. To further increase utilization of this heterologous pathway, a modified NADP⁺-utilizing *E. coli* PDH variant (PDHm) (Bocanegra et al., 1993) was constructed and introduced to

simultaneously resupply NADPH and acetyl-CoA levels (Figure 1).

The impact of the cofactor preference of PDHm and PDH was first characterized by measuring the NADPH/NADP⁺ ratios for base strain BYt+pXP843 (carrying an empty vector), strain BYt+pJCT-PDH, and strain BYt+pJCT-PDHm during batch cultivation. The ratios measured at 24 and 48 h are shown in Figure 2. Formation of active PDH complex was limited by expression of the *lpd1* gene from the *S. cerevisiae* late-phase *ADH2* promoter that is only active after glucose depletion (t >12 h). At 12 h, the three strains all had similar ratios (data not shown). However, at 24 h, the strain expressing the modified PDH (PDHm) had an increased cofactor ratio, with NADPH/NADP⁺ ratio 3.7-fold greater than the base strain and 2.2-fold greater than the strain expressing the native *E. coli* PDH. The modified PDH thus increased NADPH availability in the cell. By 48 h, the strains had similar ratios again, indicating routine utilization of NADPH as a reducing agent during stationary phase. Observing these differences well after glucose is depleted and during ethanol utilization indicates the PDH complex has a sufficient supply of pyruvate. This could be traced to respiratory metabolism and ethanol breakdown to acetate following the diauxic shift (Galdieri et al., 2010). As acetate is further assimilated to acetyl-CoA in both the cytosol and mitochondria, respiratory function commits acetyl-CoA to both the glyoxylate and TCA cycles where oxaloacetate formation becomes important (Chen et al., 2012; Haurie et al., 2001). Pyruvate can then be replenished during ethanol-grown conditions by one of two paths: direct synthesis from phosphoenolpyruvate formed from oxaloacetate, or direct synthesis from malate which is an intermediate metabolite of the glyoxylate and TCA cycles (Boles et al., 1998). This well-characterized carbon flow is also consistent with our observation that high NADPH levels are present in our engineered system, as NADPH regulates glyoxylate function under these conditions (Satrústegui et al., 1983; Ying,

2008).

Expression of the *E. coli* PDH complex may also improve acetyl-CoA levels as this pathway supplements the native yeast pyruvate dehydrogenase bypass pathway. With *in vivo* PDH activity indirectly verified by the improved cofactor ratios, the levels of acetyl-CoA were then measured for base strain BYt+pXP843 (empty vector), strain BYt+pJCT-PDH, and strain BYt+pJCT-PDHm during batch culture (Figure 3). At 24 h, the strains expressing PDH and PDHm showed 2.3-fold and 2.4-fold increases in acetyl-CoA, respectively, similar to observations at 12 h (data not shown). By 48 h the PDH overexpression system (pJCT-PDH, Figure 3) had a 58% reduction in acetyl-CoA levels relative to the base strain. This may be attributed to the NAD^+ consumption associated with the native *E. coli* complex and corresponding cofactor imbalance (Chowdhury et al., 2014). Recent computational studies demonstrated the importance of cofactor preference when introducing the PDH complex in *S. cerevisiae* for TAL synthesis (Chowdhury et al., 2014), and our experimental results corroborate this requirement. While PDH overexpression can improve acetyl-CoA levels, introducing a resupply of reducing equivalents via the NADPH-generating variant (PDHm) is more beneficial, resulting in increases in both the $\text{NADPH}/\text{NADP}^+$ ratio and acetyl-CoA levels.

3.2 NADPH driving force further increases cofactor and acetyl-CoA pools

Introduction of the NADPH-generating PDH variant (PDHm) alone was capable of increasing flux toward acetyl-CoA. Elimination of the pentose phosphate pathway (the major NADPH-generating pathway in yeast) via deletion of *ZWF1* (encoding glucose-6-phosphate dehydrogenase) should further improve utilization of this new route for acetyl-CoA, as the new pathway will now be a major source of NADPH for the cell (Figure 1).

Therefore, $\text{NADPH}/\text{NADP}^+$ and acetyl-CoA levels were measured for *ZWF1* knockout

strains BYt $\Delta zwf1$ +pXP843 (empty vector) and BYt $\Delta zwf1$ +pJCT-PDHm, and compared with the strains with an intact *ZWF1* (Figures 2 and 3). Deletion of *ZWF1* had limited effect on the NADPH/NADP⁺ ratio. This was not surprising due to the various known alternate routes for generating NADPH in *S. cerevisiae* (Grabowska and Chelstowska, 2003). However, the coupled strain ($\Delta zwf1$ + PDHm expression) had a 2.9-fold higher cofactor ratio level than the base strain at 12 h (data not shown). By 24 h, the NADPH/NADP⁺ ratio for the coupled strain was over 10-fold that of the base strain (Figure 2). Another notable outcome of this coupled strain was the observed change in acetyl-CoA levels (Figure 3). All strains with PDH overexpression (pJCT-PDH, pJCT-PDHm, and $\Delta zwf1$ +pJCT-PDHm) exhibited over 2.2 to 2.4-fold increases in acetyl-CoA after 24 h relative to the base strain. However, coupled strain BYt $\Delta zwf1$ +pJCT-PDHm was the only one that maintained high acetyl-CoA levels at 48 h. This indicates that a large change in yeast cofactor and acetyl-CoA levels takes place only when a driving force (knockout of the pentose phosphate pathway) has been implemented. The increased levels of NADPH and acetyl-CoA can be applied toward the biosynthesis of polyketides or other acetyl-CoA derived molecules.

3.3 Improved polyketide levels using PDHm in the cofactor-deficient strain

The overexpression of PDHm in the $\Delta zwf1$ strain increased both NADPH and acetyl-CoA availability, with acetyl-CoA levels higher at late stages of the cultivation. To determine the effect on polyketide synthesis, we transformed these previously tested strains with pXP842-2PS, carrying the *G. hybrida g2ps1* (encoding 2-pyrone synthase; 2-PS) under the late-phase *ADH2* promoter. TAL levels were measured after 48 h of batch cultivation (Figure 4). Co-expression of 2-PS with the native *E. coli* PDH generated 0.32 g/L TAL, a 30% increase relative to the base yeast strain. This was the smallest increase of all strains tested, indicating that the cofactor

imbalance introduced with the NAD⁺-consuming PDH may limit the possible improvement. Co-expression of 2-PS with the modified *E. coli* PDH (PDHm), resulted in a 3-fold increase in titer to 0.75 g/L TAL. To create a driving force for acetyl-CoA, the *ZWF1* deletion blocking the major NADPH-generating pathway was important (Figure 3), and should lead to increased TAL production. This $\Delta zwf1$ modification alone was previously shown to result in TAL increases, as pentose biosynthesis is a significant carbon drain from glycolysis (Cardenas and Da Silva, 2014). In the current study, knocking out *ZWF1* alone resulted in a 2.6-fold improvement over the base strain TAL. Expression of the native *E. coli* PDH in the $\Delta zwf1$ strain gave no significant increase in TAL levels relative to the $\Delta zwf1$ strain alone. However, coupling the $\Delta zwf1$ strain with PDHm overexpression increased TAL levels an additional 70% relative to BYt $\Delta zwf1$, and 4.5-fold higher relative to the base yeast strain. This supports the computational modeling by Chowdhury et al. (2014). Therefore, introduction of this biological driving force for acetyl-CoA production via PDHm improved the acetyl-CoA pools (Figure 3), and thus increased TAL synthesis (Figure 4) in late batch culture. This strategy may prove even more useful for polyketides that utilize NADPH for their synthesis, and where an even greater NADPH pool is required.

3.4 Eliminating mitochondrial precursor transport pathways

Our overall aim was to direct carbon toward cytosolic acetyl-CoA for polyketide production. In *S. cerevisiae*, various shuttling events involving acetyl-CoA and pyruvate can limit the availability of this metabolite. Therefore, we considered two main routes, the mitochondrial pyruvate carrier (MPC) and the carnitine shuttle system, as well as the common mitochondrial porins (Figure 5). For the MPC, gene products Mpc1, Mpc2, and Mpc3 form Mpc1/Mpc2 and Mpc1/Mpc3 complexes (Mpc2 and Mpc3 are paralogs), part of the active transport mechanism (Herzig et al., 2012). Using the MPC, pyruvate transport into the

mitochondrial matrix and subsequent conversion by decarboxylation commits this carbon flow toward mitochondrial acetyl-CoA (Figure 5). Replenishing pyruvate will be important to effectively use our engineered PDHm platform. Similarly, cytosolic acetyl-CoA can be transported to the mitochondria via the carnitine shuttle system (Franken et al., 2008) instead of being utilized in the cytosol (Figure 5). Therefore, limiting transport of pyruvate and acetyl-CoA from the cytoplasm while minimizing deleterious effects on the cell may be an effective means of increasing polyketide production.

We first identified a group of eight proteins involved in pyruvate and acetyl-CoA transport and conversion (Figure 5), and evaluated the effects of their single gene knockouts on both growth and TAL production. Seven of the proteins are in pathways that facilitate transport of various metabolites across the outer membrane by porins (Por1, Por2), specific transport of pyruvate from the intermembrane space into the mitochondrial matrix (Mpc1, Mpc2, Mpc3), and transport of acetyl-CoA from the cytosol into the intermembrane space (Yat1, Yat2). The knockout strains were transformed with pXP842-2PS and samples were taken at 48 h. Por1 and Por2 are responsible for the mitochondrial permeability of charged solutes including pyruvate, ATP, and phosphate by forming voltage-dependent, anion-selective channels (VDACs) in the outer membrane (Blachly-Dyson et al., 1997). Due to differences in their activity, the $\Delta por1$ and $\Delta por2$ strains produced very different levels of TAL (Figure 6). While $\Delta por2$ increased TAL 2.5-fold to 0.62 g/L, $\Delta por1$ nearly abolished product formation with a 94% reduction in titer relative to the parent strain. This is consistent with reports that Por1 maintains mitochondrial osmotic stability (Galganska et al., 2008). Por2 is not associated with this activity, and instead represses Sod1 (superoxide dismutase) that in turn alleviates respiration repression. Additionally, $\Delta por1$ has been found to suffer a growth defect due to reduced respiratory function on non-fermentable

carbon, in contrast to the increase under the same conditions for $\Delta por2$ (Dihanich et al., 1987; Sánchez et al., 2001). This is consistent with the growth observed for the culture; the $\Delta por1$ strain reached a final density equal to one-third (4.2 g DCW/L) that of the base strain (Figure 6).

The disruption of each subunit in the mitochondrial pyruvate carrier (Mpc1, Mpc2, Mpc3) also resulted in different polyketide levels. The $\Delta mpc1$ strain showed no significant effect on TAL titer relative to the base strain, while $\Delta mpc2$ and $\Delta mpc3$ strains provided 3.4-fold and 1.9-fold increases in TAL titer, respectively. Final cell densities were 86%, 89%, and 74% of the base strain for $\Delta mpc1$, $\Delta mpc2$, and $\Delta mpc3$, respectively. The Mpc1p/Mpc3p complex is necessary for retaining strong respiration and its expression is closely tied to stress responses (Timón-Gómez et al., 2013), consistent with the data showing the lower cell density for the $\Delta mpc3$ strain. The data therefore indicates an intact Mpc1/Mpc3 pyruvate carrier and disruption of the Mpc1/Mpc2 complex supports respiratory function while likely increasing cytosolic pyruvate levels leading to the significant improvement in TAL synthesis. The ability to convert pyruvate to acetyl-CoA in the mitochondria is eliminated by deleting *PDA1* (Figure 5); this deletion results in no detectable pyruvate decarboxylase activity (Gey et al., 2008). The $BY\Delta pdal$ strain produced >2-fold more TAL than the base yeast strain (0.58 g/L). This indicates that eliminating this pathway can improve titers of acetyl-CoA-derived compounds like TAL.

For cytosolic acetyl-CoA transport to the mitochondria, the first two committed steps from the carnitine shuttle system were evaluated. Yat1 and Yat2 are responsible for committing acetyl-CoA to the carnitine shuttle system and transporting it to the mitochondrial matrix, respectively (Franken et al., 2008; Swiegers et al., 2001) (Figure 5). In the $\Delta yat1$ strain, a 94% drop in TAL was observed, while in the $\Delta yat2$ strain, TAL levels increased over 2-fold relative

to the base strain (Figure 6). The data indicates that the cell is much more tolerant of eliminating the cytosolic Yat2 than the mitochondrial Yat1; the mitochondrial Yat1 is responsible for shuttling acetyl-CoA from other compartments like the peroxisome and may be more critical for proper cell function. In addition, Yat2 is highly active when ethanol is used as the carbon source (Franken et al., 2008; Swiegers et al., 2001), the same time that TAL synthesis is occurring with the *ADH2* promoter, and thus deleting this function may be advantageous. We did not consider a *CAT2* deletion strain (Figure 5) as Cat2 has activity in both the mitochondria and the peroxisome (Franken et al., 2008).

3.5 Combining transport gene disruptions with engineered NADPH driving force

From the eight genes tested for disruption, four were chosen for combination to further reduce the flow of pyruvate and acetyl-CoA transport into the mitochondria. With the single knockout strains, significant improvements in TAL levels were observed by disrupting $\Delta por2$, $\Delta mpc2$, $\Delta pdal1$, and $\Delta yat2$. Therefore, we deleted all four genes in a single strain and measured TAL levels (Figure 7, Panels A-C). Cultivation of strain BYt $\Delta por2$ yielded 0.62 g/L TAL with a reduction in biomass of 10% relative to the base strain. Knockout of *MPC2* further elevated titers to 0.98 g/L TAL, while also reducing biomass by an additional 20%. Further interventions had no negative effect on biomass, and incorporation of $\Delta pdal1$ and $\Delta yat2$ led to additional titer increases to 1.27 and 1.33 g/L, corresponding to a final yield on glucose of 0.13 g/g (28% of theoretical yield). The diminishing improvements observed with the sequential knockouts suggest that other pathways are now limiting the production of TAL. This strain was then transformed with pJCT-PDHm to determine if coupling PDHm expression with the transport knockouts would further increase TAL levels. However, no significant increase was observed.

To also introduce a NADPH driving force, the four deletions were incorporated in a

$\Delta zwfI$ strain. TAL levels were measured after 48 h of batch culture (Figure 7, Panels D-F). Plasmid stability did not change significantly even in this highly engineered strain. For both BYt and BYtB $\Delta zwfI\Delta por2\Delta mpc2\Delta pda1\Delta yat2$, 61-67% of the cells retained both plasmids after 48 hours of growth (Table S3). As shown previously (Figure 4), deletion of *ZWF1* alone led to increased TAL levels, likely due to the improved carbon flux through glycolysis. Similar increases were seen for the strains with the sequential transport knockouts; a 2-fold improvement was achieved by incorporating this four-gene disruption ($\Delta por2\Delta mpc2\Delta pda1\Delta yat2$) into the $\Delta zwfI$ strain. However, in this case, subsequent transformation with pJCT-PDHm led to a further significant increase in specific TAL production and in titer ($p<0.001$). The combination of approaches led to a final titer of 1.6 g/L TAL, corresponding to 35% of the theoretical yield on glucose (0.16 g/g), the highest reported value for batch fermentation (Figure 8). These novel strategies thus proved effective in maintaining high pyruvate and acetyl-CoA levels in the cytosol, which was in turn used for polyketide formation.

4. Conclusions

S. cerevisiae has been shown to be amenable to significant metabolic rewiring for producing acetyl-CoA derived compounds. In this study, we created a biological driving force that could increase acetyl-CoA pools applicable toward producing polyketides. Characterization of the modified NADPH-generating *E. coli* PDH (PDHm) relative to the native *E. coli* PDH indicated that NADPH synthesis was beneficial even though TAL biosynthesis does not require it directly. Coupling this with a $\Delta zwf1$ strain (to create additional driving force for acetyl-CoA synthesis) led to TAL levels 4.4-fold higher than the parent strain. This improvement was attributed to increased NADPH/NADP⁺ and acetyl-CoA levels, up to 10-fold and 3-fold higher than in the parent. To further increase the availability of acetyl-CoA for polyketide synthesis, gene deletions were used to reduce transport of acetyl-CoA and pyruvate into the mitochondria. The single gene deletions in *POR2*, *MPC2*, *PDA1*, and *YAT2* each provided over a 3-fold increase in titer (up to 0.84 g/L) relative to the parent strain. Coupling these four in a single knockout strain ($\Delta por2\Delta mpc2\Delta pda1\Delta yat2$) provided increased titer to 1.33 g/L. Introducing this four-gene disruption into the coupled $\Delta zwf1$ / pJCT-PDHm strain provided an additional 50% improvement in TAL titer to 1.6 g/L. This is 35% of the theoretical yield on glucose (0.16 g/g), the highest reported value for batch cultivation. This coupled strategy illustrates a promising step toward improving strain development for synthesis of acetyl-CoA derived compounds. Additional features can also be incorporated to further enhance acetyl-CoA pools, including reduced byproduct formation (Cardenas and Da Silva, 2014), enzyme engineering (Cardenas, 2015; Tang et al., 2013), and downregulation of competing essential pathways such as fatty acid biosynthesis.

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6. References

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Figure Captions

Figure 1: General engineering strategy applied for introducing biological driving forces. (A) Yeast relies on the pentose phosphate biosynthetic pathway (*PP Pathway*) as the major route for NADPH generation. To make acetyl-CoA in the cytoplasm, the native pyruvate dehydrogenase bypass (*PDH bypass*, green arrows) converts pyruvate to acetyl-CoA in three steps, requiring ATP. The acetyl-CoA can then be used for polyketide synthesis. (B) Introduction of the *E. coli* PDH (*EcPDH*) enhances pyruvate conversion toward acetyl-CoA using a protein complex (red arrow). (C) Our engineered cofactor-coupled pathway blocks flux through the pentose phosphate pathway (crossed out), and replaces *EcPDH* with the modified *E. coli* pyruvate dehydrogenase complex (*EcPDHm*, blue arrow) to produce acetyl-CoA and create a new NADPH supply.

Figure 2: NADPH/NADP⁺ ratios for BYt strains harboring the native *E. coli* PDH (pJCT-PDH), the NADPH-generating variant PDH (pJCT-PDHm), and/or the $\Delta zwf1$ gene knockout. Values at 24 h (hatched) and 48 h (solid) correspond to mean values $\pm$ SEM (n=2 independent experiments).

Figure 3: Acetyl-CoA levels for BYt strains harboring the native *E. coli* PDH (pJCT-PDH), the NADPH-generating variant PDH (pJCT-PDHm), and/or the $\Delta zwf1$ gene knockout. Values at 24 h (hatched) and 48 h (solid) correspond to mean values $\pm$ SEM (n=2 independent experiments).

Figure 4: TAL titers (g/L) after 48 cultivation for BYt strains harboring the native *E. coli* PDH (pJCT-PDH), the NADPH-generating variant PDH (pJCT-PDHm), and/or the $\Delta zwf1$ gene knockout. All strains carry pXP842-2PS for TAL synthesis. Values correspond to mean values $\pm$ SEM (n=6 independent experiments).

Figure 5: Metabolic pathways related to the transport and utilization of pyruvate and acetyl-CoA, the starter metabolite for subsequent TAL formation (catalyzed by 2-PS). Pathways highlighted include the major native NADPH biosynthesis pathway (Zwf1), mitochondrial pyruvate carrier (MPC) system (Mpc1/Mpc2 and Mpc1/Mpc3), mitochondrial porins (Por1 and Por2), the mitochondrial yeast PDH (Lat1/Pda1/Pdb1/Pdx1), and carnitine shuttle system (Yat1, Yat2, Crc1, Cat2). Reactions chosen for disruption in this study have been crossed out.

Figure 6: TAL titers (g/L, Panel A), specific TAL levels (g/g DCW, Panel B), and biomass (g DCW/L, Panel C) after 48 h cultivation of strains with single gene knockouts in pathways for the mitochondrial transport and utilization of pyruvate and acetyl CoA. All strains carry pXP842-2PS for TAL synthesis. Bars represent mean values $\pm$ SEM (n=6 independent experiments).

Figure 7: TAL titers (g/L), specific levels (g/g DCW), and biomass (g DCW/L) in strains carrying pJCT-PDHm engineered for transport alone ($\Delta por2\Delta mpc2\Delta pda1\Delta yat2$, Panels A-C), or in combination with $\Delta zwf1$ (Panels D-F), after 48 h cultivation. All strains carry pXP842-2PS for TAL synthesis. Bars represent mean values $\pm$ SEM (n=6 independent experiments).

Figure 8: Final titer and yield summary for TAL production in engineered *S. cerevisiae* strains. Volumetric titer (g/L), specific production (cell basis; g/g DCW), and corresponding yield on glucose (g/g) with percent theoretical yield are shown.

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7

Figure 8

Table 1. List of plasmids and strains

Plasmids	Description	Source
pXP842	2μ vector, <i>ADH2</i> promoter, <i>CYC1</i> terminator, <i>URA3</i> selectable marker	Shen et al. (2012)
pXP843	2μ vector, <i>ADH2</i> promoter, <i>CYC1</i> terminator, <i>TRP1</i> selectable marker	Leber and Da Silva (2014)
pXP842-2PS	pXP218 harboring the <i>g2ps1</i> gene encoding <i>G. hybrida</i> 2-pyrone synthase	Cardenas and Da Silva (2014)
pJCT-PDH	2μ vector, <i>ADH2p-lpd1-ADH2t</i> , <i>HXT7p-aceE-CYC1t</i> , <i>ADH1Mp-aceF-ADH2t</i> , <i>TRP1</i> selectable marker	This study
pJCT-PDHm	2μ vector, <i>ADH2p-lpd1m-ADH2t</i> , <i>HXT7p-aceE-CYC1t</i> , <i>ADH1Mp-aceF-ADH2t</i> , <i>TRP1</i> selectable marker	This study
Strains	Description	Source
BY4741	<i>MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0</i>	Open Biosystems
BY4741Δ <i>trp1</i> (BYt)	BY4741 <i>trp1::KanMX</i>	Open Biosystems
BYΔ <i>por1</i>	BY4741 <i>por1::KanMX</i>	Open Biosystems
BYΔ <i>por2</i>	BY4741 <i>por2::KanMX</i>	Open Biosystems
BYΔ <i>mpc1</i>	BY4741 <i>mpc1::KanMX</i>	Open Biosystems
BYΔ <i>mpc2</i>	BY4741 <i>mpc2::KanMX</i>	Open Biosystems
BYΔ <i>mpc3</i>	BY4741 <i>mpc3::KanMX</i>	Open Biosystems
BYΔ <i>pda1</i>	BY4741 <i>pda1::KanMX</i>	Open Biosystems
BYΔ <i>yat1</i>	BY4741 <i>yat1::KanMX</i>	Open Biosystems
BYΔ <i>yat2</i>	BY4741 <i>yat2::KanMX</i>	Open Biosystems
BYtΔ <i>zwf1</i>	BYt <i>zwf1::MET15</i>	This study
BYtΔ <i>por2</i>	BYt <i>por2::TRP1</i>	This study
BYtΔ <i>por2</i> Δ <i>mpc2</i>	BYt <i>por2::TRP1 mpc2::MET15</i>	This study
BYtΔ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i>	BYt <i>por2::TRP1 mpc2::MET15 pda1::HIS3</i>	This study
BYtΔ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i> Δ <i>yat2</i>	BYt <i>por2::TRP1 mpc2::MET15 pda1::HIS3 yat2::LEU2</i>	This study
BYtAΔ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i> Δ <i>yat2</i>	BYt <i>por2::loxP mpc2::MET15 pda1::HIS3 yat2::LEU2</i>	This study
BYtΔ <i>zwf1</i> Δ <i>por2</i>	BYt <i>zwf1::MET15 por2::LEU2</i>	This study
BYtΔ <i>zwf1</i> Δ <i>por2</i> Δ <i>mpc2</i>	BYt <i>zwf1::MET15 por2::LEU2 mpc2::HIS3</i>	This study
BYtΔ <i>zwf1</i> Δ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i>	BYt <i>zwf1::MET15 por2::LEU2 mpc2::HIS3 pda1::TRP1</i>	This study
BYtΔ <i>zwf1</i> Δ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i> Δ <i>yat2</i>	BYt <i>zwf1::MET15 por2::LEU2 mpc2::HIS3 pda1::loxP yat2::TRP1</i>	This study
BYtBΔ <i>zwf1</i> Δ <i>por2</i> Δ <i>mpc2</i> Δ <i>pda1</i> Δ <i>yat2</i>	BYt <i>zwf1::MET15 por2::LEU2 mpc2::HIS3 pda1::loxP yat2::loxP</i>	This study