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Establishing a platform cell factory through engineering of yeast acetyl-CoA metabolism

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ABSTRACT

Production of fuels and chemicals by industrial biotechnology requires efficient, safe and flexible cell factory platforms that can be used for production of a wide range of compounds. Here we developed a platform yeast cell factory for efficient provision of acetyl-CoA that serves as precursor metabolite for a wide range of industrially interesting products. We demonstrate that the platform cell factory can be used to improve the production of α-santalene, a plant sesquiterpene that can be used as a perfume by four-fold. This strain would be a useful tool to produce a wide range of acetyl-CoA-derived products.

1. Introduction

Saccharomyces cerevisiae is the most widely used cell factory employed in the production of food and beverage products, bioethanol, vaccines, therapeutic proteins and nutraceuticals (Nevoigt, 2008). With its long term use for large-scale bioethanol production this organism is well implemented in the fermentation industry. Furthermore, *S. cerevisiae* is often the organism of choice when a new process has to be developed due to its robustness towards harsh environmental conditions, the ease of genetic manipulation, and the extensive available knowledge about its physiology and biochemistry (Nielsen and Jewett, 2008). There is therefore much interest to develop *S. cerevisiae* as a general cell factory platform. Such a platform cell factory can preferentially efficiently convert the raw material, today typically glucose/fructose derived from starch or sucrose, but in the future also pentoses derived from lignocellulose (Garcia Sanchez et al., 2010; Ha et al., 2011), into so-called precursor metabolites that are then further converted into the product of interest (Nielsen and Jewett, 2008). One of these key metabolites is acetyl-CoA that is used as precursor for the production of a wide range of industrially very interesting products including isoprenoids (mainly used as flavours and fragrances, biodiesels, antimalarial and anticancer drugs, antibiotics, rubber, dietary supplements, food ingredients and vitamins), polyketides (antibiotics, anticancer drugs and immunosuppressors), lipids (such as dietary supplements, pharmaceuticals and biodiesels), polyhydroxyalkanoates and 1-butanol (Fig. 1).

These products are produced by pathways that usually drain acetyl-CoA from the cytosol. In connection with the reconstruction of synthetic pathways it is generally desirable to position these pathways in this compartment as this will minimize issues related to product secretion. Therefore, the supply of sufficient amounts of the precursor acetyl-CoA in the cytoplasm is crucial. As illustrated in Fig. 1, acetyl-CoA is, however, produced and used in several different compartments, i.e., the cytosol, mitochondria and the peroxisomes, and in *S. cerevisiae* this metabolite cannot be transported directly between the different compartments without the carnitine/acetyl-carnitine shuttle or glyoxylate cycle. Even though *S. cerevisiae* holds all components of the carnitine transport system, it is not capable of *de novo* synthesis of carnitine (van Roermund et al., 1999) and in industrial fermentations, it would be too expensive to add this component to the medium. Thus, the carnitine shuttle is excluded as a possible route to channel acetyl-CoA between different compartments.

In yeast, acetyl-CoA in the cytosol is produced from acetate that is derived from acetaldehyde, which is formed by decarboxylation of pyruvate. Acetaldehyde can also be converted to ethanol by alcohol dehydrogenase, and during growth on glucose the majority of the glycolytic flux is directed towards ethanol due to the so-called Crabtree effect in yeast (Van Hoek et al., 1998; Vemuri et al., 2007). Besides the main alcohol dehydrogenase (Adh1p) there are several alcohol dehydrogenases that can catalyze the conversion of acetaldehyde to ethanol

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Nomenclature

PDH	pyruvate dehydrogenase
MVA	mevalonate
AcAcCoA	acetoacetyl-CoA
GYC	glyoxylate cycle
FPP	farnesyl diphosphate
Sansyn	α-santalene synthase
TCA	tricaboxylic acid
ACS	acetyl-CoA synthetase
AcCoA	acetyl-CoA

(Hazelwood et al., 2008), and it is therefore inherently difficult to eliminate ethanol production in yeast. The only strategy that has worked so far is removing pyruvate de-carboxylase activity through deletion of all three genes that encode for this activity (Flikweert et al., 1996), but this results in a strain that will require supplementation of a C_2 compound such as ethanol or acetate to the medium in order to meet the requirement for acetyl-CoA in the cytosol (needed for biosynthesis of lysine and lipids) (Flikweert et al., 1999). Obviously, such a strain cannot serve as a platform cell factory for acetyl-CoA derived products.

There have been very few reports on engineering of acetyl-CoA production in yeast. Chen and co-workers reported that overexpression of *ACS1* or *ACS2* in *S. cerevisiae*, increased intracellular acetyl-CoA levels two to five-fold (Chen et al., 2010). De Jong-Gubbels et al. reported that overexpression of these two acetyl-CoA synthetase (ACS) isoenzymes in respiring *S. cerevisiae* cells increased enzyme activity by three- to six-fold, relative to an isogenic reference strain. However, ACS overexpression did not reduce acetate production after continuous cultures were pulsed with glucose (De Jong-Gubbels et al., 1998). In another study it was shown that engineering the pyruvate dehydrogenase (PDH) bypass in *S. cerevisiae* enhanced intracellular acetyl-CoA supply and thereby increased the production of the sesquiterpene compound amorphadiene (Shiba et al., 2007). This was achieved by overproduction of the endogenous *ALD6* gene which encodes an acetaldehyde dehydrogenase, and introduction of a *Salmonella enterica* acetyl-CoA synthetase variant. However, these studies focused on acetyl-CoA synthetase and there remains a need to further increase the acetyl-CoA production, which when met should increase production of any desired acetyl-CoA derived product.

In the current study, we have chosen the production of α-santalene as a model system to demonstrate our acetyl-CoA platform cell factory. α-Santalene is a naturally occurring sesquiterpene that is used for synthesis of santalol, an important constituent of sandalwood oil. The latter is an important perfumery ingredient and also largely used for incenses and in traditional medicine (Schalk, 2009). Recently we have optimized the mevalonate (MVA) pathway and downregulated the expression of squalene synthase in a fed-batch process in which α-santalene productivity was increased by 3.4-fold (Scalcinati et al., 2012). However, the supply of intracellular acetyl-CoA may be limited, which when relieved should increase α-santalene production.

In the present study we developed a platform yeast cell factory by engineering different cellular pools of acetyl-CoA. As a case study, we evaluated this platform yeast cell factory for the production of α-santalene. We demonstrated that the platform cell factory can be used to improve the production of α-santalene

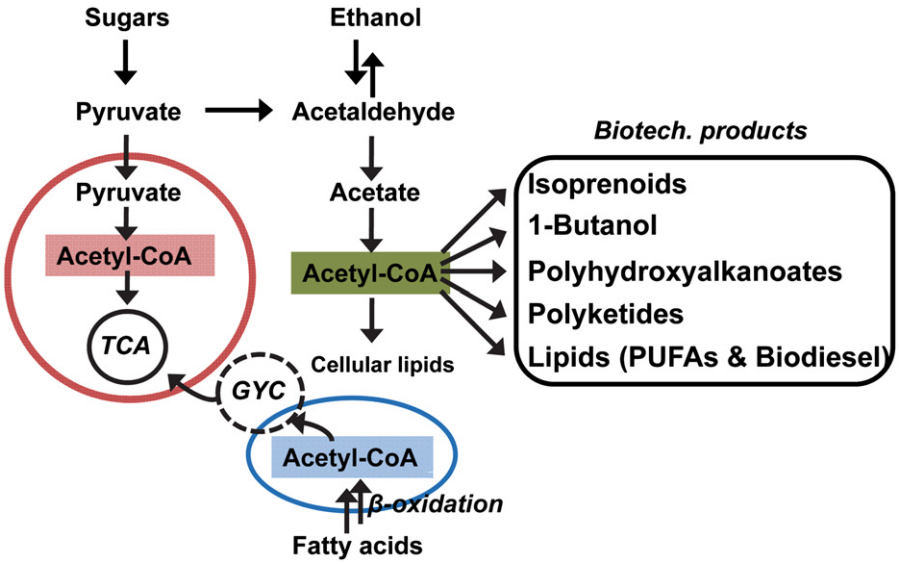

Fig. 1. *Simplified overview of acetyl-CoA metabolism in S. cerevisiae.* Acetyl-CoA is a key metabolite in three different compartments: the cytosol (marked green), the mitochondria (marked red), and the peroxisomes (marked blue). There is no direct transport of acetyl-CoA between the three compartments, and the biosynthesis of acetyl-CoA in the three compartments involves different metabolic pathways. In the mitochondria, acetyl-CoA is formed from pyruvate by the pyruvate dehydrogenase complex. In the cytosol, acetyl-CoA is formed from acetate by acetyl-CoA synthetase. In the peroxisomes, acetyl-CoA can be formed from both acetate (also by acetyl-CoA synthetase, not shown) and from fatty acids by β-oxidation. In the mitochondria, the primary fate of acetyl-CoA is oxidation via the tricarboxylic acid (TCA) cycle. Acetyl-CoA in the peroxisomes can via the glyoxylate cycle (GYC) be converted to C_4 organic acids (malate and succinate) that can be transferred to the mitochondria for oxidation via malic enzyme and the TCA cycle. The primary fate of acetyl-CoA in the cytosol is to serve as precursor for cellular lipids (fatty acids and ergosterol). Many industrially interesting biotechnological products are derived from acetyl-CoA and the biosynthesis of most of these occur in the cytosol. A platform yeast cell factory for all these products should therefore have redirection of carbon towards acetyl-CoA in the cytosol. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

four-fold. We believe the platform cell factory would be useful for production of a wide range of acetyl-CoA-derived products using yeast as a cell factory.

2. Materials and methods

2.1. Plasmid and strain construction

All plasmids and yeast strains used in this study are summarized in Table 1. pICK01 derived from a previous study (Scalcinati et al., 2012) was used for expression of *Sansyn* and *tHMG1*.The 2.1-kb *Pvu*II fragment from pSP-GM1 (Chen et al., 2012a), containing the P_{TEF1}–P_{PGK1} bidirectional promoter cassette, was cloned into the *Pvu*II sites of pESC-HIS (Agilent Technologies, Santa Clara, USA) yielding pIYC04.

All oligonucleotides used in this study are listed in Table 2. *ALD6* (GenBank NM_001183875) was PCR amplified from chromosomal DNA preparations of *S. cerevisiae* CEN.PK113-5D (*MAT*a *SUC2 MAL2-8*^{c} *ura3-52*) using primer pair 1 and 2. To ensure efficient translation, the nucleotide sequence 5′-AAAACA-3′ (Kozak sequence) (Hamilton et al., 1987) was introduced immediately upstream of the start codon of *ALD6* and the other genes used in this study. The *S. enterica* acetyl-CoA synthetase gene with the L641P mutation (Shiba et al., 2007; Starai et al., 2005), acs_{SE}^{L641P} was codon optimized and synthesized by DNA 2.0 (Menlo Park, CA, USA) for expression in yeast. A 1.5-kb *Bam*HI/*Xho*I fragment including *ALD6* and a 2.0-kb *Not*I/*Pac*I fragment containing acs_{SE}^{L641P} were sequentially inserted into the corresponding sites of pIYC04, resulting in plasmid pIYC05. To express the *ADH2* gene under the control of glucose-regulated *HXT7* promoter, a 0.6-kb promoter region of *HXT7* (GenBank NC_001136.10) was PCR amplified from CEN.PK113-5D using the primer pair 3 and 4. The amplified product was cleaved with *Bam*HI/*Fse*I and introduced into the *Bam*HI and *Fse*I sites of pSP-GM1 to replace the *PGK1* promoter, yielding plasmid pIYC06. A 1.5-kb fragment that contains the 1047-bp complete sequence of *ADH2* (GenBank NC_001145) and the 434-bp downstream sequence including its native terminator was amplified by PCR from CEN.PK113-5D using primers 5 and 6. Likewise, a 1.2-kb region of *ERG10* (GenBank NC_001148.4) from CEN.PK113-5D was PCR amplified using primers 7 and 8. A 1.5-kb *Bam*HI/*Xho*I fragment containing *ADH2* and a 1.2-kb *Spe*I/*Sac*I fragment containing *ERG10* were sequentially inserted into the corresponding sites of pIYC06, resulting in plasmid pIYC07. To overexpress *ALD6*, acs_{SE}^{L641P}, *ADH2* and *ERG10* from one plasmid, pIYC08 was constructed. A 2.0-kb fragment containing the *TEF1* promoter, *ERG10*, and the *ADH1* terminator from pIYC07 was PCR amplified using primers 9 and 10 to introduce *Mre*I/*Kpn*2I sites. The resulting DNA was cleaved with *Mre*I/*Kpn*2I and inserted into the *Mre*I/*Kpn*2I sites of pIYC05 to obtain plasmid pIYC05-1. Similarly, a cassette containing the *HXT7* promoter, *ADH2* and its native terminator from pIYC07 was PCR amplified using primers 11 and 12 to introduce two *Asc*I sites. The amplified product was digested with *Asc*I and subsequently cloned into the *Asc*I site of pIYC05-1, yielding plasmid pIYC08.

SCIYC32 was constructed by crossing strains SCIYC06 (*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 cit2*Δ, (Chen et al., 2012b)) and CEN.PK110-10C (*MAT*α *SUC2 MAL2-8*^{c} *his3-Δ1*) followed by dissection. Similarly, SCIYC33 was constructed by crossing strains SCIYC07 (*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 mls1*Δ, (Chen et al., 2012b)) and CEN.PK110-10C followed by dissection. Strain SCIYC40 was constructed by transforming plasmids pICK01 and pIYC04 into strain CEN.PK113-11C. Strain SCIYC41 was obtained by introduction of plasmids pICK01 and pIYC05 into strain CEN.PK113-11C. Plasmids pICK01 and pIYC08 were co-transformed into strain CEN.PK113-11C for the construction of SCIYC42. Similarly, these two plasmids were co-transformed into SCIYC32 and SCIYC33 resulting in SCIYC45 and SCIYC46, respectively. Three to ten colonies from each transformation were screened for the selection of the best producing transformant.

2.2. Media and growth conditions

Engineered yeast strains were selected on synthetic dextrose (SD) medium without uracil and/or histidine where appropriate (Chen et al., 2012a). *S. cerevisiae* strains were grown in defined minimal medium (Verduyn et al., 1992) with 20 g l^{-1} glucose.

To test α-santalene production from different strains, 20 ml cultures were started in 100 ml unbaffled flasks by inoculating an amount of pre-culture that resulted in a final optical density of 0.02 at 600 nm (OD_{600}). The strains were grown at 30 °C with

Table 2
Primers used in this study.

Primer no.	Sequence (5′ to 3′)
1	CGCCGGATCCAAAACAATGACTAAGCTACACTTTGAC
2	CGCGCTCGAGTTACAACTTAATTCTGACAGC
3	TATTAGGCCGGCCCCGTGGAAATGAGGGGTATGC
4	GGCCGGCGGATCCTTTTTGATTAAAATTAAAAAAACT
5	GCGCGGATCCAAAACAATGTCTATTCCAGAAACTCAA
6	GCGATCCGGAACGTCAAGACGAAAAGTGAA
7	GCCGCGACTAGTAAAACAATGTCTCAGAACGTTT ACA
8	GCGGCCCGAGCTCTCATATCTTTTCAATGACAAT
9	CTATCTTCCGGAGCACACACCATAGCTTC
10	TCTAAACGCCGGCGAATTGGAGCGACCTCATGC
11	AAACTCCTAGGCCGTGGAAATGAGGGGTA
12	GTCAAAGGCGCGCCACGTCAAGACGAAAAGT

Restriction sites are underlined.

Table 1
Strains and plasmids used in this study.

Name	Host strain		Plasmid	
	Name	Genotype	Name	Description
SCIYC40	CEN.PK113-11C[a]	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1*	pIYC04	(2μ *HIS3*)
			pICK01	P_{TEF1}–*Sansyn* P_{PGK1}–*tHMG1* (2μ *URA3*)
SCIYC41			pIYC05	P_{TEF1}–acs_{SE}^{L641P} P_{PGK1}–*ALD6* (2μ *HIS3*)
			pICK01	P_{TEF1}–*Sansyn* P_{PGK1}–*tHMG1* (2μ *URA3*)
SCIYC42			pIYC08	P_{TEF1}–acs_{SE}^{L641P} P_{PGK1}–*ALD6* P_{TEF1}–*ERG10* P_{HXT7}–*ADH2* (2μ *HIS3*)
			pICK01	P_{TEF1}–*Sansyn* P_{PGK1}–*tHMG1* (2μ *URA3*)
SCIYC45	SCIYC32	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1 cit2*Δ	pIYC08	P_{TEF1}–acs_{SE}^{L641P} P_{PGK1}–*ALD6* P_{TEF1}–*ERG10* P_{HXT7}–*ADH2* (2μ *HIS3*)
			pICK01	P_{TEF1}–*Sansyn* P_{PGK1}–*tHMG1* (2μ *URA3*)
SCIYC46	SCIYC33	*MAT*a *SUC2 MAL2-8*^{c} *ura3-52 his3-Δ1 mls1*Δ	pIYC08	P_{TEF1}–acs_{SE}^{L641P} P_{PGK1}–*ALD6* P_{TEF1}–*ERG10* P_{HXT7}–*ADH2* (2μ *HIS3*)
			pICK01	P_{TEF1}–*Sansyn* P_{PGK1}–*tHMG1* (2μ *URA3*)

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180 r.p.m. orbital shaking in defined minimal medium (Verduyn et al., 1992) with 20 g l^{-1} glucose. To prepare the pre-cultures, 15 ml culture tubes containing 5 ml of defined medium were inoculated with a single colony of strains of interest. These were cultured at 30 °C with 220 r.p.m. orbital shaking to an OD_{600} between 1 and 2. For analysis of α-santalene production, 10 % (v/v) dodecane was added to the main culture at an OD_{600} of 1 to capture α-santalene in the organic phase.

2.3. Bioreactor cultivation

Aerobic batch cultures were carried out in 1.0 l Dasgip stirrer-pro® bioreactor (DasGip, Jülich, Germany) with 0.6 l working volume. The temperature was controlled at 30 °C and the air flow rate was kept to 1 l per liquid volume per minute (1 vvm). The pH of the culture media was maintained at 5.0 by controlled addition of 2 M KOH. The agitation was settled from 600 rpm to 1200 rpm for keeping the dissolved oxygen concentration in the media above 30%. The concentration of dissolved oxygen was measured with an autoclavable polarographic oxygen electrode (Mettler Toledo, Columbus, OH, USA). The off-gas from the bioreactors was monitored for real-time determination of oxygen and carbon dioxide concentration by Dasgip fedbatch pro® gas analysis systems, equipped with off-gas analyzer 1 GA4 based on zirconium dioxide and two-beam infrared sensor (DasGip). Strains were grown in defined minimal medium with the following composition: 5.0 g l^{-1} $(NH_4)_2SO_4$; 3.0 g l^{-1} KH_2PO_4; 0.5 g l^{-1} MgSO4 · 7H2O; 1 ml l^{-1} trace metal solution (per liter, pH 4.0: EDTA (sodium salt), 15.0 g; $ZnSO_4 \cdot 7H_2O$, 0.45 g; MnCl2 · $2H_2O$, 1 g; $CoCl_2 \cdot 6H_2O$, 0.3 g; $CuSO_4 \cdot 5H_2O$, 0.3 g; $Na_2MoO_4 \cdot 2H_2O$, 0.4 g; $CaCl_2 \cdot 2H_2O$, 0.45 g; $FeSO_4 \cdot 7H_2O$, 0.3 g; H_3BO_3, 0.1 g and KI, 0.10 g). The pH of minimal medium was adjusted to 5.0 by adding 2 M NaOH and autoclaved separately from the carbon source solution. Glucose was added at a concentration of 20 g l^{-1}. Vitamin solution (per liter, pH 6.5: biotin, 0.05 g; *p*-amino benzoic acid, 0.2 g; nicotinic acid, 1 g; Ca-pantothenate, 1 g; pyridoxine-HCl, 1 g; thiamine-HCl, 1 g and myo-inositol, 25 g) was filter sterilized and aseptically added to the medium after autoclaving at the concentration of 1 ml l^{-1}. The pre-cultures were prepared using the same conditions as for shake flask cultivation. 600 ml of medium was inoculated to an OD_{600} of 0.02 from late exponential phase shake flask cultures. 10 % (v/v) dodecane was added to the main culture at an OD_{600} of 1 to capture α-santalene in the organic phase.

2.4. Analytical methods

The OD_{600} was measured throughout the cultivation. Dry cell weight (DCW) was measured by filtering 5 ml of culture broth through a 0.45 μm nitrocellulose filter and measuring the increased weight of the dry filter. Glucose, ethanol, glycerol, and acetate were measured using a Summit HPLC (Dionex, Sunnyvale, CA) with an Aminex HPX-87H column (Bio-Rad, Hercules, CA) as described before (Chen et al., 2012b). The dodecane layer was sampled and diluted in decane for the determination of α-santalene production by GC–MS as described earlier (Scalcinati et al., 2012).

3. Results

3.1. Constructing the yeast platform strain

For efficient provision of acetyl-CoA in the cytoplasm, we used a combined push-pull-block strategy (Fig. 2A). This strategy involved a push of carbon from ethanol via acetaldehyde to cytosolic acetyl-CoA and a block of further conversion of acetyl-CoA by competing pathways. The push part of the strategy involved over-expression of the endogenous *ADH2* gene, encoding alcohol dehydrogenase, by using the glucose-regulatable *HXT7* promoter, as well as constitutive over-expression of *ALD6*, encoding NADP-dependent aldehyde dehydrogenase, and a codon-optimized *ACS* variant (L641P) from *S. enterica* (acs_{SE}^{L641P}), encoding

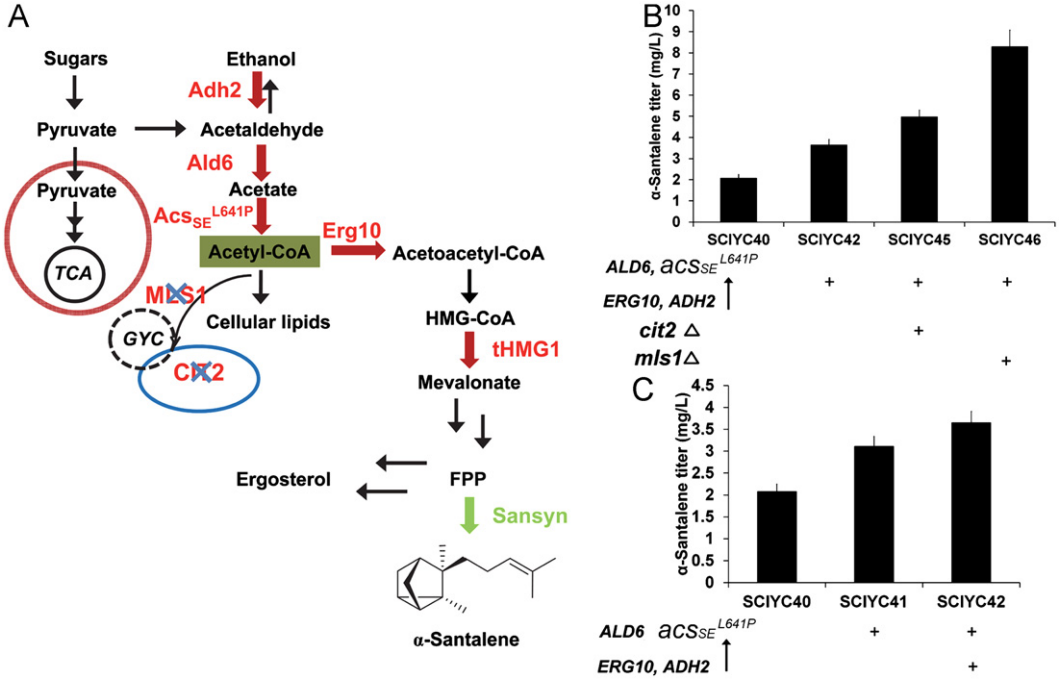

Fig. 2. *Engineering of yeast acetyl-CoA metabolism improves the production of α-santalene.* (A) Illustration of the push–pull–block strategy for improving the production of acetyl-CoA in the cytosol and schematic representation of biosynthetic pathways for production of α-santalene. The genetic modifications are shown by the thick arrows and cross signs. (B), (C) Evaluation of the yeast platform strains for α-santalene production in shake flasks. Error bars represent the standard errors of at least three replicates. tHMG1, hydroxymethylglutaryl-CoA reductase; Sansyn, α-santalene synthase.

acetyl-CoA synthetase. acs_{SE}^{L641P} contains a point mutation that prevents the enzyme from being inhibited by acetylation (Starai et al., 2005), and the use of this variant was previously demonstrated to successfully redirect flux from acetaldehyde to acetyl-CoA in the cytosol (Shiba et al., 2007). Over-expression of *ADH2* ensures that ethanol produced by Adh1p during growth on glucose can be converted back to acetaldehyde and from here further to acetyl-CoA. To ensure pulling of acetyl-CoA towards the products of interest we over-expressed *ERG10* encoding acetyl-CoA C-acetyltransferase that catalyzes the conversion of acetyl-CoA to acetoacetyl-CoA (AcAcCoA), which is a common precursor for useful products derived therefrom, such as for example terpenoids, 1-butanol and polyhydroxybutyrate. Finally, the block part of the strategy ensured reduction of carbon loss from the precursor acetyl-CoA pool and this was achieved through removing reactions that involve consumption of acetyl-CoA. This involved inhibition of two key reactions of the glyoxylate cycle (GYC), namely peroxisomal citrate synthase, encoded by *CIT2*, and cytosolic malate synthase, encoded by *MLS1*. We recently demonstrated that acetyl-CoA used by citrate synthase in the peroxisomes is formed by Acs1p from acetate and that acetyl-CoA in the cytosol can be consumed by Mls1p and hereby lead to a net conversion into C_4 organic acids that can be transported to the mitochondrion for oxidation (Chen et al., 2012b). Even though this pathway is predominantly active during growth on C_2 compounds such as ethanol and acetate, the pathway has been demonstrated to be active also during growth on glucose (Regenberg et al., 2006).

3.2. Evaluation of α-santalene production

We choose production of α-santalene as an example to evaluate the platform strain. As shown in Fig. 2A, α-santalene is derived directly in a single enzymatic step from farnesyl diphosphate (FPP), the biological precursor for sesquiterpenes. To increase the FPP production, the mevalonate pathway was deregulated by over-expression of the catalytic domain of 3-hydroxy-3-methylglutaryl-coenzyme A reductase 1 (*tHMG1*), previously demonstrated to result in an improvement of isoprenoid production in yeast (Asadollahi et al., 2010; Ro et al., 2006). The transgenic yeast strain SCIYC40 (Table 1), as reference strain, was transformed with a plasmid pICK01 containing *tHMG1* and *Sansyn* under the control of the constitutive promoters *PGK1* and *TEF1*, respectively (Scalcinati et al., 2012). In a shake-flask culture, 2.08 mg l^{-1} α-santalene was produced by the reference strain SCIYC40 after 48 h of growth (Fig. 2B). In strain SCIYC42 α-santalene production was increased to 3.65 mg l^{-1} as a result of over-expressing *ALD6*, acs_{SE}^{L641P}, *ADH2* and *ERG10* (on plasmid pIYC08) (Fig. 2B). To evaluate our strategy compared with previous work (Shiba et al., 2007) on over-expression of acs_{SE}^{L641P} and *ALD6*, we also over-expressed these two genes alone from plasmid pICK01. The results showed that additional over-expression of *ADH2* and *ERG10* resulted in a 25% increase in α-santalene titer (Fig. 2C) over the two-gene strategy. Combining the *CIT2* deletion and co-expression with plasmid pIYC08 resulted in strain SCIYC45 that was able to produce 4.98 mg l^{-1} α-santalene (Fig. 2B). Furthermore, when plasmid pIYC08 was co-expressed in an *MLS1* deletion strain, α-santalene production was increased to 8.29 mg l^{-1} (Fig. 2B, strain SCIYC46), which represents a four-fold improvement compared with the reference strain.

3.3. Linking α-santalene production with host metabolism

Manipulation of acetyl-CoA metabolism increased α-santalene production, which depends on cytosolic acetyl-CoA as precursor, suggesting an increased availability of acetyl-CoA in the cytoplasm. We therefore further evaluated the carbon fluxes around this metabolite in all constructed strains. Except for α-santalene formation, the main fluxes in C_2 metabolism are the drain of acetyl-CoA to biomass, and formation/consumption of ethanol and acetate (Fig. 3A). The physiological properties of the recombinant strains were therefore characterized in batch cultivation (Table 3). The specific growth rates of the recombinant strains on glucose were found to be similar to their reference strain, but there was a trend of decrease (to different extents) in the ethanol, acetate and biomass yield (on glucose) for all the engineered strains, while the α-santalene yield on glucose increased, with three–four fold for strains SCIYC45 and SCIYC46. Thus, it is clear that there was not only obtained a higher final titer with our engineering strategy, but the strategy also ensured a re-direction of fluxes towards the acetyl-CoA derived product α-santalene during growth on glucose in all the engineered strains (Fig. 3B).

In the ethanol phase, a significant drop in biomass yield was observed for strains SCIYC45 and SCIYC46. We have previously shown that deletion of *CIT2* or *MLS1* affects the utilization of non-fermentable carbon sources such as ethanol or acetate (Chen et al., 2012b). Thus little α-santalene is produced during the ethanol phase (Fig. 3B). However, α-santalene yield and accumulation on ethanol were enhanced about 2 times in strain SCIYC46, compared to strains containing *MLS1*, but the productivity was quite low (data not shown), since the time for complete utilization of ethanol was doubled in SCIYC46.

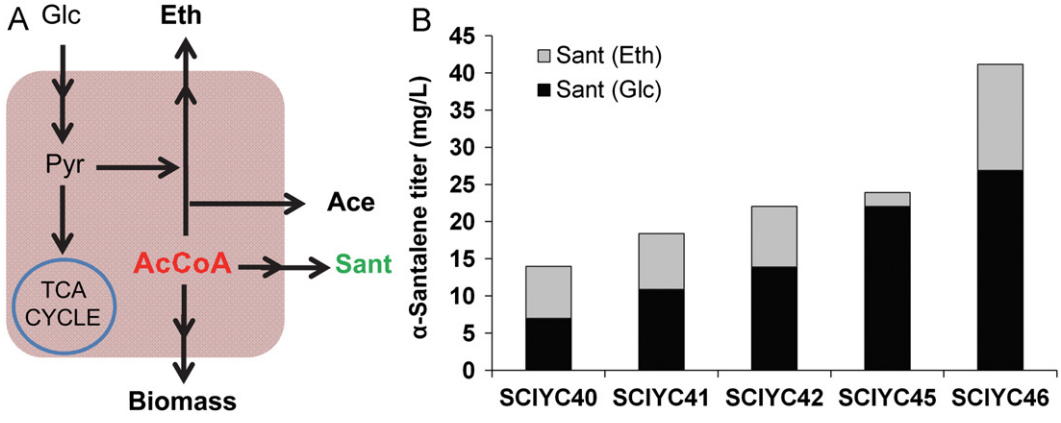

Fig. 3. *Linking α-santalene production with host metabolism.* (A), Schematic representation of endogenous pathways competing for cytosolic acetyl-CoA. AcCoA, acetyl-CoA. (B), Accumulation of α-santalene during the glucose and ethanol phase in batch cultures. Data are mean of duplicate bioreactors.

Table 3
Physiological properties of the recombinant strains.

Strains	μ_{max}[a]	Yeth/glc (mol/mol)	Yace/glc (mol/mol)	Yx/glc[b] (mol/mol)	Ysan/glc (mmol/mol)	Yx/eth[c] (mol/mol)	Ysan/eth (mmol/mol)
SCIYC40	0.24	1.35	0.05	1.13	0.26	1.30	0.18
SCIYC41	0.22	1.32	0.04	1.11	0.42	1.28	0.25
SCIYC42	0.21	1.27	0.04	1.10	0.46	1.22	0.26
SCIYC45	0.24	1.31	0.03	0.96	0.87	0.18	0.10
SCIYC46	0.23	1.24	0.04	1.06	0.98	0.22	0.52

The average value from duplicate independent cultivations is shown and in all cases, the standard error was less than 3%.

[a] Maxium specific growth rate (h^{-1}) on glucose.
[b] Yield of biomass from glucose.
[c] Yield of biomass from ethanol.

4. Discussion

Acetyl-CoA is an essential intermediate in numerous metabolic pathways, and is also typically considered as a building block for the biosynthesis of many industrially interesting products (Fig. 1). There have been many reasons for increasing the exploitation of *S. cerevisiae* in industrial biotechnology (Nevoigt, 2008). It is relatively tolerant to low pH values and high sugar concentrations, which lowers the risk of contaminations in industrial fermentations. In addition, it is fairly resistant to inhibitors present in biomass hydrolysates. Furthermore, for expression of pathways containing P450 enzymes yeast is generally preferred compared to bacterial cell factories. Thus, there is growing interest in developing yeast cell factories for production of acetyl-CoA derived products. Acetyl-CoA metabolism in yeast is quite complex and this metabolite is present in different compartments and cannot be directly transported between these compartments. In this study, we therefore engineered the acetyl-CoA metabolism in *S. cerevisiae* for efficient provision of this precursor metabolite for sesquiterpene production.

Like many other eukaryotic cells *S. cerevisiae* does not contain ATP:citrate lyase. Without the carnitine shuttle the activation of acetate is therefore the only route providing acetyl-CoA in the cytosol. During growth on glucose the high glycolytic flux is directed towards ethanol formation, meaning high flux from pyruvate to acetaldehyde. This is consistent with high activity levels of pyruvate decarboxylase, even under glucose-limited and respiratory conditions (Pronk et al., 1996). In order to redirect flux towards acetyl-CoA it is therefore necessary to redirect the carbon from acetaldehyde to acetyl-CoA in the cytosolic compartment. Overexpressing endogenous *ALD6* and introducing a codon-optimized acs_{SE}^{L641P} was here found to result in an increase in α-santalene production, which is consistent with a previous report (Shiba et al., 2007). We further combined this strategy with overexpression of *ADH2* and *ERG10* as this would allow for pulling in ethanol towards acetyl-CoA and also push acetyl-CoA towards acetoacetyl-CoA. Of all alcohol dehydrogenases in *S. cerevisiae*, only Adh2p is responsible for oxidizing ethanol to acetaldehyde (Russell et al., 1983) and the expression of *ADH2* is repressed in the presence of glucose by several hundred-fold (Blumberg et al., 1988). To ensure that ethanol, which is formed as a product of overflow metabolism, was converted back to acetaldehyde, *ADH2* was overexpressed under the control of the *HXT7* promoter, which is generally highly active at lower concentrations of glucose (Diderich et al., 1999). To pull the carbon from the cytosolic acetyl-CoA pool towards the products of interest *ERG10* encoding acetyl-CoA C-acetyltransferase, which catalyzes the condensation of two molecules of acetyl-CoA to form acetoacetyl-CoA, was overexpressed under the control of *TEF1* promoter. As a result, α-santalene production was further increased by 25% with our four-gene overexpression strategy, compared to previous strategies which used overexpression of acetaldehyde dehydrogenase and introduction of the *S. enterica* acetyl-CoA synthetase variant alone (Shiba et al., 2007). From our physiological characterization we showed that our push and pull strategy in strain SCIYC42 enabled a flux redirection towards acetyl-CoA as the ethanol yield on glucose and the biomass yield on ethanol were decreased compared to strain SCIYC41 using the two-gene strategy. This observation indicated that the carbon flux was redirected into cytosolic acetyl-CoA and further into acetoacetyl-CoA.

To minimize the drain of acetyl-CoA in the cytoplasm to be used for the tricaboxylic acid (TCA) cycle, we deleted *CIT2* and *MLS1*, respectively, which encode the key enzymes in the glyoxylate cycle and ensure formation of C_4 organic acids that can be transported into the mitochondria for oxidation. In a former study, we have shown that deletion of *CIT2* or *MLS1* could affect the utilization of non-fermentable carbon sources, such as ethanol and acetate (Chen et al., 2012b). In strains SCIYC45 and SCIYC46, the biomass yields on ethanol were found significantly decreased, whereas in strain SCIYC46 the α-santalene yield on ethanol was shown to be two--three fold increased, compared to the hosts without *MLS1* deletion (Table 3). This could be due to the fact that *MLS1* deletion resulted in an attenuation of glyoxylate cycle activity (Chen et al., 2012b).

5. Conclusion

The development of efficient cell factories for production of chemicals and fuels is generally time consuming and often considered to be a limitation in the advancement of industrial biotechnology. In order to be able to faster develop novel cell factories it will be valuable to develop platform cell factories that have their metabolism engineered such that there is an efficient conversion of the carbon source to metabolites that serve as precursors for industrially relevant products. Here we have developed such a conceptual yeast platform strain capable of efficient provision of acetyl-CoA that is a precursor for a wide range of industrially interesting products. We have demonstrated that this yeast platform cell factory can be used for improving the production of α-santalene by four-fold. This platform cell factory can be used for a wide range of acetyl-CoA derived products, as shown in Fig.1, e.g., polyhydroxybutyrate that can be used as a biodegradable plastic and ployketides. We believe that the strategies presented here will form the basis for further development towards industrial processes for production of chemicals based on microbial fermentation.

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