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Engineering of the pyruvate dehydrogenase bypass in *Saccharomyces cerevisiae* for high-level production of isoprenoids

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Abstract

Amorphadiene, a sesquiterpene precursor to the anti-malarial drug artemisinin, is synthesized by the cyclization of farnesyl pyrophosphate (FPP). *Saccharomyces cerevisiae* produces FPP through the mevalonate pathway using acetyl-CoA as a starting compound. In order to enhance the supply of acetyl-CoA to the mevalonate pathway and achieve high-level production of amorphadiene, we engineered the pyruvate dehydrogenase bypass in *S. cerevisiae*. Overproduction of acetaldehyde dehydrogenase and introduction of a *Salmonella enterica* acetyl-CoA synthetase variant increased the carbon flux into the mevalonate pathway resulting in increased amorphadiene production. This work will be generally applicable to the production of a broad range of isoprenoids in yeast.

Keywords: Acetaldehyde dehydrogenase; Acetyl-CoA synthetase; Amorphadiene; Isoprenoids

1. Introduction

Isoprenoids are among the most diverse groups of compounds synthesized by biological systems and are important in maintaining membrane fluidity, electron transport, protein prenylation, and in cellular and organismal development. They also have industrial importance as fragrances, essential oils, and antibacterial and antifungal agents (McCaskill and Croteau, 1997, 1998). Because they are often produced naturally in small quantities, purification from biological sources suffers from low yields and impurities and results in consumption of large amounts of natural resources. Furthermore, because of the complexity of these molecules, the chemical syntheses of isoprenoids are inherently difficult and expensive and result in relatively low yields (Avery et al., 1992; Danishefsky et al., 1996; Nicolaou et al., 1997). For these reasons, engineering metabolic pathways to produce large quantities of complex isoprenoids in a tractable biological host is an attractive alternative to extraction from environmental sources or chemical syntheses. Recently, high-level, in vivo production of isoprenoids in *Escherichia coli* was demonstrated. Engineering the expression of a synthetic amorpha-4,11-diene synthase gene and the mevalonate pathway from *Saccharomyces cerevisiae* in *E. coli* led to the production of large quantities of amorphadiene, the sesquiterpene olefin precursor to the potent anti-malarial drug artemisinin (Martin et al., 2003).

Sterol biosynthesis via the mevalonate pathway in *S. cerevisiae* involves more than 20 distinct reactions initiated with acetyl-CoA and proceeding though farnesyl pyrophosphate (FPP), a branch-point intermediate for sesquiterpenes and sterols (Daum et al., 1998). Several terpene synthases from plants or other organisms have

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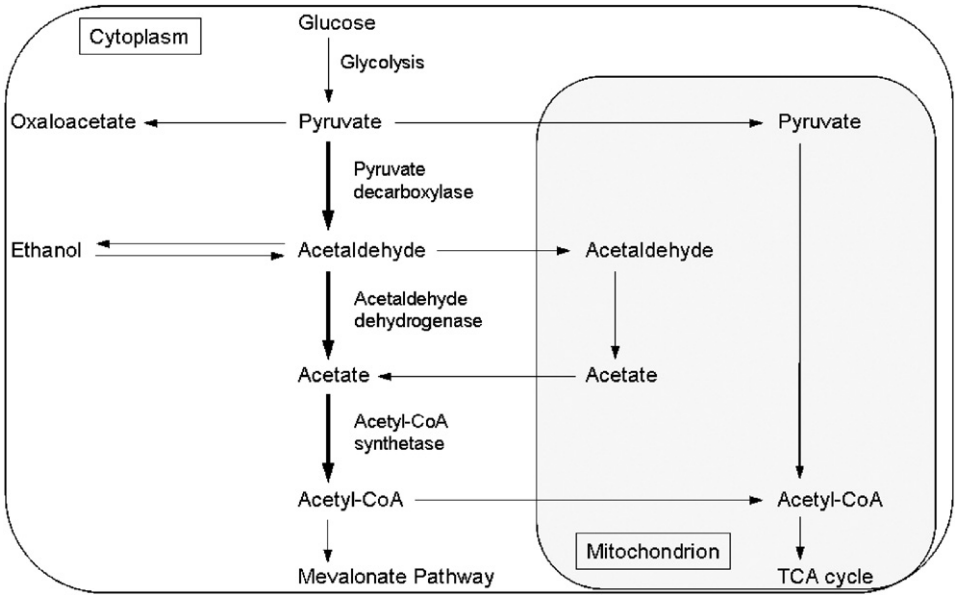

Fig. 1. Schematic representation of the pyruvate dehydrogenase bypass.

been successfully expressed in yeast, rendering it an attractive host for heterologous isoprenoid production (Dejong et al., 2005; Jackson et al., 2003; Misawa and Shimada, 1997). By overproducing key enzymes in the mevalonate pathway, overproducing a transcription factor responsible for regulating expression of several genes in the mevalonate pathway, and down-regulating squalene synthesis, we were able to increase the production of amorphadiene many fold (Ro et al., 2006). However, there remains a bottleneck in the supply of acetyl-CoA to the mevalonate pathway, which when relieved should increase production of any desired terpene.

To enhance the supply of acetyl-CoA to the mevalonate pathway and achieve high-level production of amorphadiene in yeast, we focused on a primary metabolic pathway, the pyruvate dehydrogenase bypass (Fig. 1). The pyruvate dehydrogenase bypass converts pyruvate into acetyl-CoA by the action of pyruvate decarboxylase, cytosolic acetaldehyde dehydrogenase, and acetyl-CoA synthetase (Pronk et al., 1996). Here we demonstrate that engineering the pyruvate dehydrogenase bypass is effective for high-level production of isoprenoids in yeast.

2. Materials and methods

2.1. Strains

S. cerevisiae EPY213 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* pRS425ADS P_{GAL1}-*tHMGR* P_{GAL1}-*upc2-1 erg9::* P_{MET3}-*ERG9*) and EPY224 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0* pRS425ADS P_{GAL1}-*tHMGR* P_{GAL1}-*upc2-1 erg9::* P_{MET3}-*ERG9* P_{GAL1}-*tHMGR* P_{GAL1}-*ERG20*) are amorphadiene-producing strains engineered for high expression of mevalonate pathway genes (Ro et al., 2006). These strains were used as the hosts for all subsequent transformations.

Table 1
Yeast strains constructed for this study

Strain	Host	Plasmid	Promoter-gene
PDB101	EPY213	pRS426ALD6 (multicopy)	*GAL1-ALD6*
PDB102	EPY213	pδ-ALD6 (integration)	*GAL1-ALD6*
PDB103	EPY213	pRS426ACS1 (multicopy)	*GAL1-ACS1*
PDB104	EPY213	pδ-ACS1 (integration)	*GAL1-ACS1*
PDB105	EPY213	pESC-ALD6-ACS1 (multicopy)	*GAL1-ALD6*, *GAL10-ACS1*
PDB106	EPY213	pESC-ALD6-SEacs (multicopy)	*GAL1-ALD6*, *GAL10-acs*
PDB107	EPY213	pESC-ALD6-SEacs^{L641P} (multicopy)	*GAL1-ALD6*, *GAL10-acs*^{L641P}
PDB108	EPY224	pESC-ALD6-SEacs^{L641P} (multicopy)	*GAL1-ALD6*, *GAL10-acs*^{L641P}

In EPY213, amorphadiene synthase gene (*ADS*) was expressed under the control of the *GAL1* promoter on a multicopy plasmid. A truncated, soluble form of HMG-CoA reductase gene (t*HMGR*) and *upc2-1*, a semi-dominant mutant allele that enhances the activity of *UPC2* (a global transcription factor regulating the biosynthesis of sterols in *S. cerevisiae*), were expressed under the control of the *GAL1* promoter by integration into the genome. The *ERG9* promoter was swapped with a methionine-repressible promoter (P_{MET3}) to down-regulate the expression of *ERG9* encoding squalene synthase. EPY224 differs from EPY213 in that *ERG20*, encoding FPP synthase, and an additional t*HMGR* under the control of the *GAL1* promoter were integrated into the genome.

All yeast strains used and constructed in this study are summarized in Table 1. *E. coli* DH10B and DH5α (Invitrogen, Carlsbad, CA) were used as the host for the propagation and manipulation of plasmids.

2.2. Plasmid construction

To overexpress genes, we constructed multicopy plasmids derived from pRS426 (Mumberg et al., 1994)

Table 2
Primer pairs used in this study

Primer-pair	Sequence
ALD6-1	5′-CCCGGATCCAACAATGACTAAGCTACACTTTGA-3′
	5′-GGGCCCGGGTTACAACTTAATTCTGACAG-3′
ALD6-2	5′-CCCGGATCCAACAATGACTAAGCTACACTTTGA-3′
	5′-GGGGCTAGCTTACAACTTAATTCTGACAG-3′
ACS1-1	5′-CCCACTAGTAACAATGTCGCCCTCTGCCGTACA-3′
	5′-GGGAAGCTTTTACAACTTGACCGAATCAA-3′
ACS1-2	5′-CCCGCGGCCGCAACAATGTCGCCCTCTGCCGTACA-3′
	5′-GGGTTAATTAATTACAACTTGACCGAATCAA-3′
acs-1	5′-CCCGCGGCCGCAACAATGAGCCAAACACATAAACACGCC-3′
	5′-GGGTTAATTAATTATGACGGCATCGCGATGGCCTG-3′
acs-2	5′-TCCTGGCGTGGTGGAGAAACCGCTCGAAGAGA-3′
	5′-GTTTCTCCACCACGCCAGGATCGGCGAGAG-3′
ACT1	5′-ATGGATTCTGGTATGTTCTAGCGC-3′
	5′-TTAGAAACACTTGTGGTGAACGAT-3′

Underlined residue identifies the mutagenic nucleotide.

and pESC-URA (Stratagene, La Jolla, CA) and integration plasmids derived from pδ-UB (Lee and Da Silva, 1997). The primer pairs used in this study are listed in Table 2. *ALD6*, encoding cytosolic acetaldehyde dehydrogenase (Meaden et al., 1997), was isolated by PCR from chromosomal DNA preparations of *S. cerevisiae* JRY1593 (Gardner et al., 1998) using the primer pair ALD6-1 and cloned into the *Bam*HI and *Sma*I sites of pRS-SacII-DX (Ro et al., 2006). In the resulting multicopy plasmid pRS426ALD6, *ALD6* is under the control of the *GAL1* promoter. A 2.3-kb *Sac*II fragment containing the *GAL1* promoter, *ALD6*, and the *CYC1* terminator from pRS426ALD6 was inserted into the *Sac*II site of pδ-UB to obtain the integration plasmid pδ-ALD6.

ALD6 was also amplified using primer another pair ALD6-2 and cloned into the *Bam*HI and *Nhe*I sites of pESC-URA to obtain the plasmid pESC-ALD6.

ACS1, encoding acetyl-CoA synthetase (De Virgilio et al., 1992), was isolated by PCR from chromosomal DNA preparations of *S. cerevisiae* JRY1593 using the primer pair ACS1-1 and cloned into the *Hin*dIII and *Spe*I sites of pRS-SacII-DX. In the resulting multicopy plasmid pRS426ACS1, *ACS1* is under the control of the *GAL1* promoter. A 2.9-kb *Sac*II fragment containing the *GAL1* promoter, *ACS1*, and the *CYC1* terminator from pRS426ACS1 was inserted into the *Sac*II site of pδ-UB to obtain the integration vector pδ-ACS1.

ACS1 was also amplified using another primer pair ACS1-2 and cloned into the *Not*I and *Pac*I sites of pESC-ALD6. In the resulting multicopy plasmid pESC-ALD6-ACS1, *ALD6* and *ACS1* are under the control of the *GAL1* and the *GAL10* promoters, respectively.

The *Salmonella* acetyl-CoA synthetase gene (*acs*, gi:16767525) was isolated by PCR from chromosomal DNA preparations of *Salmonella enterica* TR6583 (Lee et al., 2005) using the primer pair acs-1 and cloned into the *Not*I and *Pac*I sites of pESC-ALD6. In the resulting multicopy plasmid pESC-ALD6-SEacs, *ALD6* and *acs* are under the control of the *GAL1* and the *GAL10* promoters, respectively.

The Gene Tailor Site-Directed Mutagenesis system (Invitrogen) was employed to introduce amino acid substitution into *acs*. The primer pair acs-2 was used to introduce the L641P mutation into the *S. enterica* acetyl-CoA synthetase. The sequence change was confirmed by DNA sequence analysis, and the mutated *acs* was introduced into the *Not*I and *Pac*I sites of pESC-ALD6 to obtain the plasmid pESC-ALD6-SEacs^{L641P}. In this plasmid, the mutated *acs* is under the control of the *GAL10* promoter.

2.3. DNA manipulation

Standard procedures were followed for general DNA manipulations (Sambrook et al., 1989). Yeast transformation was carried out using the lithium-acetate method (Rose et al., 1990). Plasmids pδ-ALD6 and pδ-ACS1 were linearized at the unique *Xho*I site in the δ-sequence before transformation.

2.4. Medium and cultivation

For the cultivation of all yeast strains, SD medium containing 2% galactose as a sole carbon source was prepared with the composition described previously (Ro et al., 2006). Preculture in test tubes containing 5 mL of SD medium was performed at 30 °C for 3–4 days on a rotary shaker (200 rpm). After preculture, cells were inoculated into 50 mL of fresh SD medium in a 250 mL Erlenmeyer flask to an OD_{600} (optical density measured at 600 nm) of 0.05 and cultivated at 30 °C for 1–8 days on a rotary shaker (200 rpm). All flasks contained 5 mL dodecane to sequester amorphadiene. The dodecane layer was sampled and diluted in ethyl acetate for determination of amorphadiene production by GC-MS.

2.5. GC-MS analysis of amorphadiene

Amorphadiene production by the various strains was measured by GC-MS as described previously (Martin et al., 2001) by scanning for two ions, the molecular ion (204 m/z) and the 189 m/z ion. Amorphadiene concentrations were converted to caryophyllene equivalents using a caryophyllene standard curve based on the relative abundance of ions 189 and 204 m/z to their total ions. The sesquiterpene caryophyllene was purchased from Sigma-Aldrich (Saint Louis, MO).

2.6. Preparation of cell extracts and enzyme assays

Samples of cultures were harvested at $5000 \times g$ and washed twice in 10 mM potassium phosphate buffer (pH 7.5). Cells were suspended in 100 mM potassium phosphate buffer (pH 7.5) containing 2 mM $MgCl_2$ and 1 mM dithiothreitol and broken using FastProtein Red Matrix (Qbiogen, Carlsbad, CA). Debris was removed by centrifugation at $15{,}000 \times g$ and the supernatant was used as a cell extract. Enzyme activities were assayed immediately after the preparation of cell extracts. The protein concentration was determined with the DC protein assay kit (BioRad, Richmond, CA) using bovine serum albumin as a standard. The $NADP^+$-dependent acetaldehyde dehydrogenase activity was determined as described previously (Postma et al., 1989) except that $MgCl_2$ was used instead of KCl. The acetyl-CoA synthetase activity was determined as described previously (Van den Berg et al., 1996). 1 mU represents 1 nmol of substrate converted per min.

2.7. RT-PCR analysis

Total RNA of *S. cerevisiae* was extracted and isolated with the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacture's instructions. Rapid amplification of transcripts was carried out with SuperScript III One-Step RT-PCR System (Invitrogen) according to the instructions provided. Primer pairs ALD6-2, ACS1-2, and ACT1 (Table 2) were used for *ALD6*, *ACS1* and *ACT1* transcripts, respectively. Aliquots (10 ng) of total RNA were used as templates for RT-PCR. Reaction conditions were: 94 °C for 15 s; 55 °C (*ALD6* and *ACS1*) or 50 °C (*ACT1*) for 30 s; and 68 °C for 2 min, for 25 cycles (*ALD6* and *ACS1*) or 40 cycles (*ACT1*). The amplified cDNAs were analyzed by electrophoresis, stained with SYBR Green, and visualized by densitometry (Gel Doc 2000, Bio-Rad).

2.8. Other methods

SDS-PAGE was performed as described previously (Laemmli, 1970) and proteins were visualized using Coomassie Blue staining. Acetic acid and ethanol were analyzed by enzymatic assays (R-Biopharm, Darmstadt, Germany). Mevalonate was measured as mevalonolactone by GC-MS after acidification and extraction with ethyl acetate (Woollen et al., 2001).

3. Results

3.1. Overexpression of ALD6

Yeast catabolizes glucose to ethanol even under aerobic conditions. Pyruvate decarboxylase (Pdc) is the first enzyme in the pyruvate dehydrogenase bypass and is also the key enzyme of alcoholic fermentation. Pdc converts pyruvate to acetaldehyde, which is the metabolic branch point between ethanol and acetyl-coA production (Fig. 1). In *S. cerevisiae*, Pdc is present at high levels even during glucose-limited and respiratory growth (Pronk et al., 1996). It has been reported that the Michaelis–Menten constant of acetaldehyde dehydrogenase for acetaldehyde is two orders-of-magnitude lower than that of alcohol dehydrogenase (Postma et al., 1989). In order to enhance the carbon flux into the pyruvate dehydrogenase bypass, we first overexpressed *ALD6*, encoding cytosolic acetaldehyde dehydrogenase, under the control of a galactose inducible promoter.

S. cerevisiae PDB101 harboring *ALD6* on a multicopy plasmid and PDB102 containing an integrated copy of *ALD6* showed 19 and 4.6 times higher activity than that of EPY213, respectively (Table 3). PDB101 accumulated seven-fold more acetate than EPY213 (Fig. 2(a)). Overexpression of *ALD6* in each strain decreased cell mass, resulting in a decrease in amorphadiene production by 30–40%. When ethanol levels were compared, both PDB101 and PDB102 produced less than EPY213 (Fig. 2(b)). These results suggest that overexpression of *ALD6* caused a change in the carbon flux from ethanol to acetate.

3.2. Overexpression of ACS1

In *S. cerevisiae*, acetyl-CoA synthetase is encoded by two genes, *ACS1* and *ACS2*. Van den Berg compared the kinetic parameters of the two enzymes and demonstrated that Acs1p had 3 times higher Vmax and thirty times lower Km for acetate than those of Acs2p (Van den Berg et al., 1996). Therefore, *ACS1* was chosen for overexpression in our study. The strains engineered to overexpress *ACS1*

Table 3
Specific activity of acetaldehyde dehydrogenase in cell extracts of strains overexpressing *ALD6*

Strain	Specific activity (mU/mg-protein)
PDB101 (EPY213/pRS426ALD6)	29.6 ± 7.0
PDB102 (EPY213/pδ-ALD6)	7.4 ± 1.2
PDB105 (EPY213/pESC-ALD6-ACS1)	62.3 ± 7.0

Yeast strains were cultivated for 2 days in SD medium. Cell extracts were used for enzyme assay. 1 mU represents 1 nmole of acetaldehyde converted per min.

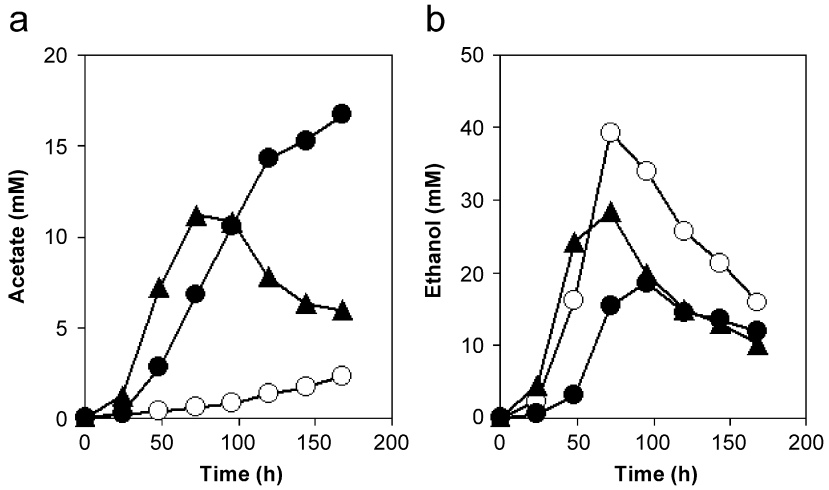

Fig. 2. Time course of acetate (a) and ethanol (b) in the medium when overexpressing *ALD6*. Strain EPY213 was transformed with a multicopy plasmid pRS426ALD6 or an integration plasmid pδ-ALD6 to overexpress *ALD6*. Yeast strains were cultivated for 7 days in SD medium. The culture supernatants were used for the analysis of metabolites. Symbols: open circles, EPY213; closed circles, PDB101 (EPY213/pRS426ALD6); closed triangles, PDB102 (EPY213/pδ-ALD6). These data are the results from a single experiment but are representative of data collected from three independent experiments.

Table 4
Specific activity of acetyl-CoA synthetase in cell extracts of strains overexpressing *ACS1*, *Salmonella acs*, or its variant (L641P)

Strain	Specific activity (mU/mg-protein)
EPY213	11.7±0.5
PDB101 (EPY213/pRS426ALD6)	10.8±0.6
PDB103 (EPY213/pRS426ACS1)	26.3±7.2
PDB104 (EPY213/pδ-ACS1)	22.5±1.0
PDB105 (EPY213/pESC-ALD6-ACS1)	14.5±0.3
PDB106 (EPY213/pESC-ALD6-SEacs)	23.9±2.9
PDB107 (EPY213/pESC-ALD6-SEacs^{L641P})	47.0±0.8

Yeast strains were cultivated for 2 days in SD medium. Cell extracts were used for enzyme assay. 1 mU represents 1 nmole of acetate converted per min.

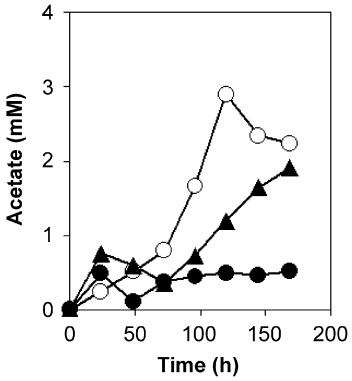

Fig. 3. Time course of acetate in the medium when overexpressing *ACS1*. Strain EPY213 was transformed with a multicopy plasmid pRS426ACS1 or an integration plasmid pδ-ACS1 to overexpress *ACS1*. Yeast strains were cultivated for 7 days in SD medium. The culture supernatants were assayed for acetate. Symbols: open circles, EPY213; closed circles, PDB103 (EPY213/pRS426ACS1); closed triangles, PDB104 (EPY213/pδ-ACS1). These data are the results from a single experiment but are representative of data collected from three independent experiments.

(PDB103 and PDB104) showed 2.2 and 1.9 times higher activity than that of EPY213, respectively (Table 4). PDB103 harboring *ACS1* on a multicopy plasmid exhibited lower acetate levels compared with EPY213 (Fig. 3). Overexpression of *ACS1* in each strain did not affect cell growth and increased amorphadiene production by 8–23%. When excess acetate was fed to a culture of PDB103, the strain consumed the acetate completely, and amorphadiene production increased (data not shown). These results suggest that more acetate was converted to acetyl-CoA when *ACS1* was overexpressed, and this contributed to an increase in amorphadiene production.

3.3. Overexpression of both ALD6 and ACS1

Overexpression of *ALD6* increased acetate production. In contrast, overexpression of *ACS1* reduced acetate production and increased amorphadiene production. Based on these results, we overexpressed both genes simultaneously on a multicopy plasmid using a bidirectional *GAL1*/*GAL10* promoter (PDB105) and investigated the effect on amorphadiene production. Although PDB105 showed higher amorphadiene production than PDB101, it did not exceed the production level of EPY213 (Fig. 4(a)). Surprisingly, more acetate accumulated in the medium of PDB105 (Fig. 4(b)) and a decrease in cell mass was observed. PDB105 had only 1.1 times higher amorphadiene productivity (production level/OD) than that of EPY213 or PDB101. To investigate the reason why overexpression of both genes did not improve the production level, enzyme activities, expression levels, and protein levels were analyzed. PDB105 showed about 40 times higher acetaldehyde dehydrogenase activity than that of EPY213 (Table 3). On the other hand, the acetyl-CoA synthetase

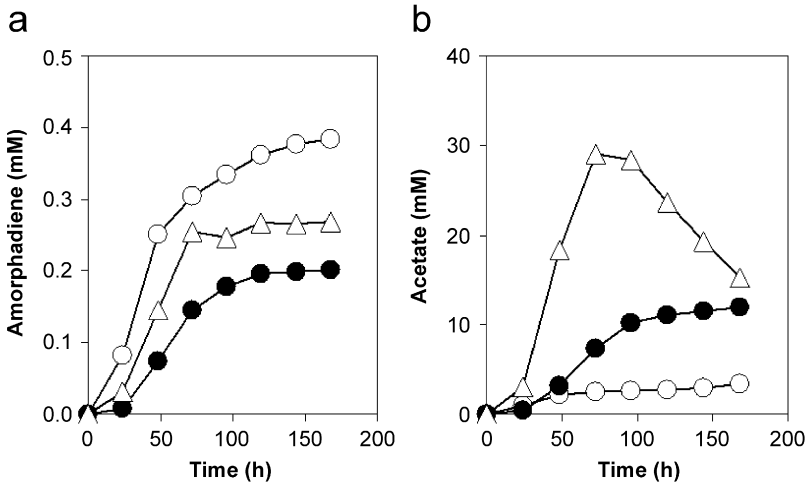

Fig. 4. Time course of amorphadiene (a) and acetate (b) in the medium when overexpressing both *ALD6* and *ACS1*. Strain EPY213 was transformed with a multicopy plasmid pESC-ALD6-ACS1 to overexpress both *ALD6* and *ACS1*. Yeast strains were cultivated for 7 days in SD medium. The dodecane layer and the culture supernatants were used for analysis of amorphadiene and acetate, respectively. Symbols: open circles, EPY213; closed circles, PDB101 (EPY213/pRS426ALD6); open triangles, PDB105 (EPY213/pESC-ALD6-ACS1). These data are the results from a single experiment but are representative of data collected from three independent experiments.

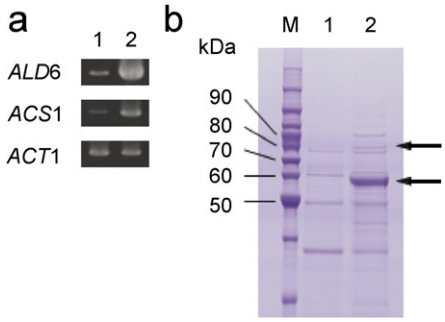

Fig. 5. Expression (a) and protein levels (b) when overexpressing both *ALD6* and *ACS1*. (a) Total RNA was prepared from yeast cells grown in SD medium for 24 h. The amplification of *ACT1* was used as a positive control. The amplification of *ALD6* without reverse transcriptase was used as a negative control and did not generate any bands (data not shown). Lane 1, EPY213; lane 2, PDB105 (EPY213/pESC-ALD6-ACS1). (b) Yeast strains were cultivated for 2 days in SD medium. The cell extracts equivalents to 200 ng of total proteins were electrophoresed. Lane M, standard proteins with sizes indicated (in kDa); lane 1, EPY213; lane 2, PDB105 (EPY213/pESC-ALD6-ACS1). The upper and lower arrows indicate the position of Acs1p and Ald6p, respectively.

activity of PDB105 was only 20% higher than that of EPY213 (Table 4). This imbalance in the enzyme activities caused an accumulation of acetate. Overexpression of both genes (PDB105) resulted in a significant increase in the corresponding mRNA and protein levels compared with EPY213 (Fig. 5). Therefore, the disparity between the increase in acetaldehyde dehydrogenase activity and the increase in acetyl-CoA synthetase activity did not appear to be due to expression problems.

3.4. Overexpression of Salmonella acs variant

When *ALD6* was overexpressed on a multicopy or an integration plasmid, acetaldehyde dehydrogenase activity increased significantly, and acetate accumulation depended on the enzyme activity. Compared with the increase in acetaldehyde dehydrogenase activity observed, acetyl-CoA synthetase activity did not increase substantially in response to *ACS1* overexpression.

In the prokaryote *Salmonella*, acetyl-CoA synthetase (Acs) is post-translationally regulated. The protein acetyltransferase acetylates Acs at lysine 609, rendering the enzyme inactive. The NAD-dependent Sir2 protein deacetylase (CobB) activates Acs via removal of the inhibitory acetyl group (Starai and Escalante-Semerena, 2004a). Because the amino acid sequences around the acetylation site between *Salmonella* and yeast are well conserved (Fig. 6), we considered the possibility that yeast acetyl-CoA synthetase is subject to post-translational regulation like *Salmonella*. It has been reported that substituting proline for leucine at position 641 on *Salmonella enterica* Acs prevents the acetylation of Acs and maintains it in its active state (Starai et al., 2005). Based on these facts and our results, we constructed a multicopy plasmid to co-express *S. enterica acs* or its variant (L641P) with *ALD6* and investigated the effect on enzyme activity and amorphadiene production.

Overexpression of *Salmonella* wild type *acs* (PDB106) increased acetyl-CoA synthetase activity compared to EPY213 and PDB105 (Table 4); however, amorphadiene and acetate levels were almost the same as those of PDB105 (data not shown). When *Salmonella acs* variant (L641P) was overexpressed with *ALD6* (PDB107), acetyl-CoA synthetase activity increased 4.0 and 3.2 times higher than that of EPY213 and PDB105 (Table 4). The amorphadiene production level of PDB107 was higher than that of PDB101 and PDB105, and the acetate concentration in the culture medium decreased to 7.6 mM, which corresponds to 54% and 44% of that of

Fig. 6. Alignment of the C-terminal ~50 amino acids of acetyl-CoA synthetase from *S. enterica* and *S. cerevisiae*. The location of the acetylation site (Lys-609 on *S. enterica* Acs) is indicated by an asterisk. The Leu-641 of *S. enterica* Acs, which is critical for the acetylation of residue Lys-609, is indicated by the pound symbol.

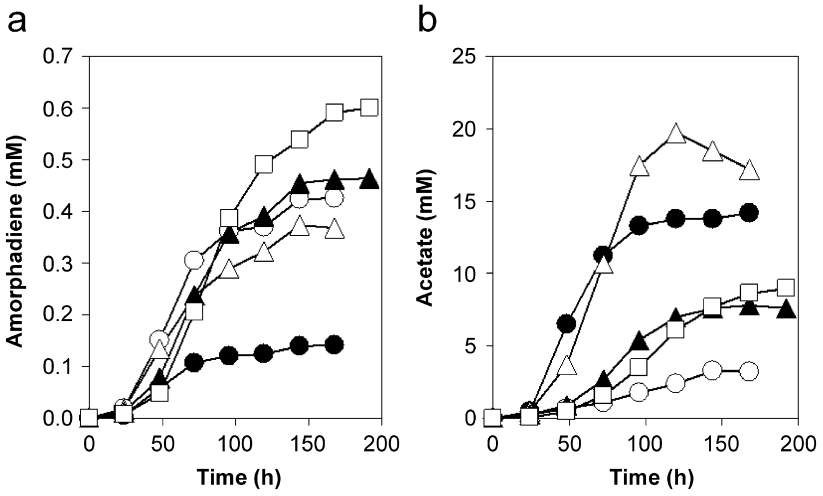

Fig. 7. Time course of amorphadiene (a) and acetate (b) in the medium when overexpressing both *ALD6* and the *Salmonella acs* variant (L641P). Strain EPY213 and strain EPY224 were transformed with a multicopy plasmid pESC-ALD6-SEacs^{L641P} to overexpress both *ALD6* and the *Salmonella acs* variant. Yeast strains were cultivated for 7 or 8 days in SD medium. The dodecane layer and the culture supernatants were used for the analysis of amorphadiene and acetate, respectively. Symbols: open circles, EPY213; closed circles, PDB101 (EPY213/pRS426ALD6); open triangles, PDB105 (EPY213/pESC-ALD6-ACS1); closed triangles, PDB107 (EPY213/pESC-ALD6-SEacs^{L641P}); open squares, PDB108 (EPY224/pESC-ALD6-SEacs^{L641P}). These data are the results from a single experiment but are representative of data collected from three independent experiments.

Table 5
Comparison of amorphadiene and mevalonate production among various strains

Strain	Amorphadiene (mM)	Mevalonate (mM)	Amorphadiene (mM/OD_{600})	Mevalonate (mM/OD_{600})
EPY213	0.356±0.001	1.78±0.06	0.127±0.003	0.64±0.02
PDB107 (EPY213/pESC-ALD6-SEacs^{L641P})	0.435±0.009	2.52±0.17	0.224±0.008	1.30±0.09
EPY224	0.493±0.010	2.04±0.06	0.197±0.002	0.82±0.03
PDB108 (EPY224/pESC-ALD6-SEacs^{L641P})	0.604±0.005	2.55±0.17	0.370±0.003	1.56±0.10

Yeast strains were cultivated for 7 or 8 days in SD medium. The dodecane layer and the culture broth were used for the analysis of amorphadiene and mevalonate, respectively.

PDB101 and PDB105, respectively (Fig. 7). PBD107 had 1.8 times higher amorphadiene productivity (production level/OD) than that of PDB101 (Table 5).

In order to further increase the production level, *Salmonella acs* variant (L641P) and *ALD6* were overexpressed in strain EPY224, which differs from EPY213 in that it has been engineered for FPP synthase overexpression and carries an additional expression cassette for a truncated form of HMG-CoA reductase (Ro et al., 2006). This strain (PDB108) achieved the highest amorphadiene production level of any strain tested, over 0.6 mM (about 120 mg/L). Productivity in PDB108 increased 1.9-fold over EPY224 (Fig. 7, Table 5). More mevalonate was observed in the cultures of PDB107 and PDB108 than in those of the parental strains. These results suggest that engineering of the pyruvate dehydrogenase bypass enhanced the carbon flux into the mevalonate pathway and this contributed to an increase of amorphadiene production.

4. Discussion

There have been no reports on engineering the pyruvate dehydrogenase bypass for improved isoprenoid production in yeast. In *E. coli*, Alper et al. showed that the knockout of *aceE* encoding pyruvate dehydrogenase improved lycopene

production, which suggests that redirecting precursor pyruvate to the non-mevalonate pathway is effective for isoprenoid production (Alper et al., 2005). We attempted to increase precursor availability by enhancing the supply of acetyl-CoA to the mevalonate pathway and demonstrated here that overproduction of acetaldehyde dehydrogenase and introduction of a heterologous acetyl-CoA synthetase variant can have a considerable impact on isoprenoid production in yeast.

Acetyl-CoA synthetase is present in most organisms from bacteria to humans, and complex regulatory systems control the expression of acetyl-CoA synthetase gene as a function of carbon flux, including a post-translational regulation implemented by the NAD^+/sirtuin-dependent protein acetylation/deacetylation system (Luong et al., 2000; Starai et al., 2002; Starai and Escalante-Semerena, 2004b). In *S. cerevisiae*, two structural genes, *ACS1* and *ACS2*, have been identified, which differ in many respects. Expression of *ACS1* is repressed by glucose and derepressed by ethanol or acetate (De Virgilio et al., 1992). On the other hand, *ACS2* is essential for growth on glucose and is expressed constitutively (Van den Berg et al., 1996; Van den Berg and Steensma, 1995). De Jong-Gubbels et al. reported that overproduction of the acetyl-CoA synthetase isoenzyme in a respiring cell does not reduce the acetate level when steady-state cultures are pulsed with glucose (De Jong-Gubbels et al., 1998). In our experiment, strain EPY213 accumulated one-fourth the acetate compared with De-Jong-Gubbels' control strain, and overexpression of *ACS1* in EPY213 led to a reduction in acetate production. In another study, Acs1p is reportedly subject to glucose-induced degradation (De Jong-Gubbels et al., 1997); we used galactose as a sole carbon source. We conclude that the culture conditions and the physiological state are crucial to the activity and stability of acetyl-CoA synthetase, which ultimately affects the acetate and amorphadiene levels.

The carboxy-terminal tripeptide VKL of Acs1p is similar to the peroxisome-targeting signal SKL motif, which indicates that Acs1p may be located in peroxisomes (McNew and Goodman, 1996). The other acetyl-CoA synthetase isoenzyme (Acs2p) is considered to be localized in cytoplasm (Van den Berg and Steensma, 1995). In the light of the localization, we also tried to overexpress *ACS2* with *ALD6*. However, acetyl-CoA synthetase activity and amorphadiene production remained unchanged (data not shown). We considered the possibility that acetyl-CoA synthetase is post-translationally regulated in yeast and chose to bypass the regulation by engineering expression of *Salmonella acs* in *S. cerevisiae*. Although introducing *Salmonella* wild-type *acs* increased enzyme activity, acetate accumulated and amorphadiene production did not increase. One possible explanation for these results may be that the *Salmonella* Acs has a higher Km for its substrates than the yeast Acs1p.

Another possibility is that the L641P mutation affected the in vivo activity of the enzyme. It was reported that the L641P mutation of wild type Acs abolishes the site for post-translational modification (acetylation at lysine residue), and thus the Acs^{L641P} remains active without being inactivated by acetylation; however, the substitution of the regulatory amino acid by point mutation decreases Acs specific activity about 3-fold in in vitro assays, indicating that the enzyme stability and/or kinetic properties of Acs are affected by this mutation (Starai et al., 2005). Further detailed analyses of the enzyme kinetics and stability were not pursued in previous and the current studies, and it is not understood how this L641P mutation alters the intrinsic catalytic properties of Acs. Consequently, it is also difficult to project the in vivo activity of Acs^{L641P} in yeast where a physiologically relevant level of substrate (acetate) is present. Nonetheless, our data here demonstrated that the expression of Acs^{L641P}, insensitive to the post-translation inactivation, in yeast is sufficient to compensate for the potentially weakened Acs activity by L641P mutation. Further studies of the enzyme kinetics are necessary to clarify the impact of L641P mutation on the Acs catalytic properties, and ultimately it could be possible to identify a mutant Acs which abolishes the post-translational inactivation but provides the same or higher Acs enzyme activity.

Engineering the pyruvate dehydrogenase bypass increased the mevalonate and amorphadiene levels. This was more remarkable in PDB108, because hydroxymethylglutaryl-CoA reductase (HMGR) has been identified as a rate-limiting step in the mevalonate pathway (Hampton et al., 1996). The difference in the levels of acetate for PDB101 and PDB107 was 6.6 mM, while the difference in the levels of amorphadiene for those two strains was 0.32 mM (Fig. 7). Nine moles of acetate are required to synthesize one mole of amorphadiene. From this ratio, we can infer that 2.9 mM acetate was converted to 0.32 mM amorphadiene in PDB107. Accordingly, the 0.32 mM additional amorphadiene produced by PDB107 compared to PDB101 accounts for 44% of the 6.6 mM difference in acetate levels. By the same reasoning, the additional amorphadiene production in PDB108 compared to PDB101 accounts for 79% of the difference in acetate levels. These data also suggest that engineering of the pyruvate dehydrogenase bypass is effective for enhancing the flux into the mevalonate pathway and high-level production of amorphadiene. A comparison of amorphadiene production and productivity (on an OD basis) between the engineered strains (PDB107 and PDB108) and the control strains (EPY213 and EPY224) suggests that the engineered strains have a capacity for improved production if the culture biomass is increased to the same level as the control strains. We believe this progress in engineering primary metabolism can be generally applied to production of other isoprenoids in yeast, and that further study directed to areas such as flux analysis, plasmid stability, medium assessment, and efflux from the cell may lead to further increases in production level.

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