ENVIRONMENTAL BIOTECHNOLOGY

Comparative proteomic analysis of engineered *Saccharomyces cerevisiae* with enhanced free fatty acid accumulation

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Abstract The engineered *Saccharomyces cerevisiae* strain $\triangle faa1 \triangle faa4$ [*Acot5s*] was demonstrated to accumulate more free fatty acids (FFA) previously. Here, comparative proteomic analysis was performed to get a global overview of metabolic regulation in the strain. Over 500 proteins were identified, and 82 of those proteins were found to change significantly in the engineered strains. Proteins involved in glycolysis, acetate metabolism, fatty acid synthesis, TCA cycle, glyoxylate cycle, the pentose phosphate pathway, respiration, transportation, and stress response were found to be upregulated in $\triangle faa1 \triangle faa4$ [*Acot5s*] as compared to the wild type. On the other hand, proteins involved in glycerol, ethanol, ergosterol, and cell wall synthesis were downregulated. Taken together with our metabolite analysis, our results showed that the disruption of Faa1 and Faa4 and expression of Acot5s in the engineered strain $\triangle faa1 \triangle faa4$ [*Acot5s*] not only relieved the feedback inhibition of fatty acyl-CoAs on fatty acid synthesis, but also caused a major metabolic rearrangement. The rearrangement redirected carbon flux toward the pathways which generate the essential substrates and cofactors for fatty acid synthesis, such as acetyl-CoA, ATP, and NADPH. Therefore, our results help shed light on the mechanism for the increased production of fatty acids in the engineered strains, which is useful in providing information for future studies in biofuel production.

Keywords *Saccharomyces cerevisiae* · Proteomic analysis · Acyl-CoA synthetase · Acyl-CoA thioesterase · Free fatty acids

Introduction

Microbial synthesized fatty acids have increasingly attracted attention in recent decades as a potential feedstock for biofuel production. This is because microbially synthesized fatty acids are easily converted into many fuel molecules such as alkanes, alcohols, and biodiesel through biochemical or catalytic chemical methods (Choi and Lee 2013; Howard et al. 2013; Liu et al. 2013; Steen et al. 2010). Recently, there has been great progress in engineering *Saccharomyces cerevisiae* to produce free fatty acids (FFA). It was shown that by disruption of the two main acyl-CoA synthases, Faa1 and Faa4, the yeast strain secreted 220 μmol/L (~60 mg/L) of FFA (Scharnewski et al. 2008). Besides *FAA1* and *FAA4* disruption, overexpression of *Escherichia coli* acyl-ACP thioesterase in *S. cerevisiae* yielded 207 mg/L FFA, and combined with overexpression of the fatty acid synthase (FAS) and acetyl-CoA carboxylase, resulted in the production of up to 400 mg/L FFA (Runguphan and Keasling 2014). In another study, it was demonstrated that the disruption of the β-oxidation pathway and the acyl-CoA synthetases, overexpression of different thioesterases, and enhancement of the supply of acetyl-CoA resulted in the production of more than 120 mg/L FFA (Li et al. 2014). In a latest study, it was found that disruption of the acyl-CoA synthetase genes *FAA1*, *FAA4*, and *FAT1* yielded 490 mg/L FFA, combined with disruption of the β-oxidation pathway and co-expression of lipid body forming enzyme

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DGA1 and lipase *TGL3*, 2.2 g/L FFA was produced (Leber et al. 2015).

In a previous study, we successfully increased the accumulation of FFA to ~500 mg/L in minimal medium by engineering the acyl-CoA metabolism of *S. cerevisiae*. This was done by double deletion of acyl-CoA synthases Faa1 and Faa4, and cytosolic expression of truncated version of acyl-CoA thioesterase Acot5 (Acot5s) from *Mus musculus* (Chen et al. 2014). We found a significant increase in extracellular fatty acids in the engineered strains *△faa1 △faa4* and *△faa1 △faa4* [*Acot5s*], as compared to the wild-type (WT) strain. This showed that the enzymes acyl-CoA synthetases Faa1, Faa4, and thioesterase Acot5s had a direct effect on FFA accumulation. In addition, our RT-PCR results showed the genes involved in fatty acid synthesis were de-repressed in the strains *△faa1 △faa4* and *△faa1 △faa4* [*Acot5s*], although it should be taken into account that mRNA levels do not always correlate with protein expression (Griffin et al. 2002; Ideker et al. 2001). Therefore, to understand the mechanism for the increase of FFA in the engineered strains, proteomic analysis is needed.

The regulation of the gene, protein, and metabolic network are closely connected in *S. cerevisiae* (Smedsgaard and Nielsen 2005). The enzymes involved in fatty acid synthesis and elongation are regulated on both the gene expression and protein level. In addition, compounds needed in the fatty acid synthesis process such as acetyl-CoA, ATP, and NADPH are competed for by many other cellular reactions and metabolic pathways (Tehlivets et al. 2007).

Our previous study on acyl-CoA metabolism in engineered cells has provided important insights into the regulation of fatty acid synthesis. The use of proteomic analysis will further aid the understanding of the effects of gene modification, and also enable a global overview of metabolic regulation. Hence, we conduct comparative proteomic analysis between the WT, *△faa1 △faa4*, and *△faa1 △faa4* [*Acot5s*] strains in this study. The changes in proteins involved in different metabolic pathways were identified and analyzed. Furthermore, the global metabolic changes in the engineered strains were mapped.

Materials and methods

Strain growth and cell harvest

All strains used in this study were constructed based on *S. cerevisiae* WT strain BY4741 (*MAT*a; *his3 △1*; *leu2 △0*; *met15 △0*; *ura3 △0*, EUROSCARF). As previously described (Chen et al. 2014), the control strain WT [pVTU260] was constructed by transforming the WT strain with plasmid pVTU260 (also shown as WT). The double deletion strain *△faa4 △faa1* was transformed with the plasmid pVTU260 to construct the strain *△faa4 △faa1*

[pVTU260] (also shown as *△faa4 △faa1*). The strain *△faa1 △faa4* [*Acot5s*] was constructed by transforming *△faa4 △faa1* with the plasmid pVTU260-Acot5s. All strains were streaked on YNBD-Ura agar plates (0.67 % yeast nitrogen base (with no amino acids but with ammonium sulfate, Invitrogen), 2 % dextrose, amino acid drop out (without Ura, Clonetech), and 2 % agar), and incubated at 30 °C for 2–3 days. Five milliliters of YNBD-Ura liquid medium were inoculated with a single colony and incubated overnight at 30 °C, 250 rpm. This pre-culture was then used to inoculate 50 mL YNBD-Ura liquid medium with an initial OD_{600} (optical density at 600 nm) of 0.2. OD_{600} was measured using Nano Drop 2000 (Thermo Scientific) to monitor growth status. Cell culture samples were collected at different time points. The culture supernatant and cell pellet were collected separately by centrifugation at 10,000g for 10 min at 4 °C.

Protein Extraction, Digestion, and Labeling with iTRAQ Reagents

Cells with an OD_{600} of 20 from each sample were collected, washed with PBS buffer, and re-suspended in 500 μL NP40 cell lysis buffer (Invitrogen) supplemented with 1 mM PMSF and protease inhibitor cocktail. Then, 300 μL acid-washed glass beads were added and the cells were lysed using a FastPrep®-24 Instrument (6004–500, MP Biomedicals) for 20 s, and chilled on ice for 30 s. This was repeated six times. Next, the cells were centrifuged at 12,000g for 10 min at 4 °C. The clear lysate was then transferred to new sterile microcentrifuge tubes which were pre-chilled on ice.

Protein concentration was determined by 2-D Quant Kit (GE Healthcare). Then, 100 μg of protein from each sample was cleaned up using acetone precipitation. Four volumes of ice cold acetone were added to each sample, and the protein was precipitated at −20 °C for at least 2 h. After centrifugation at 12,000g for 10 min, the acetone was removed. Then, the sample was digested and labeled using the iTRAQ Reagent Multiplex Kit (AB SCIEX) according to manufacturer's instructions. First, each protein sample was re-dissolved in 20 μL dissolution buffer and 1 μL denaturant, followed by adding 2 μL reducing reagent and incubated at 60 °C for 1 h. Then, 1 μL cysteine blocking reagent was added into each tube and incubated at room temperature for 10 min. Next, 20 μL of 0.25 μg/μL sequence-grade modified trypsin (Promega) was added into each sample. The digestion reaction was carried out at 37 °C for 12–16 h. Samples from three strains were labeled with a different iTRAQ tag, respectively. The labeled peptides were mixed together accordingly and vacuum evaporated to proper concentration.

Online 2-D Nano-LC-MS/MS analysis

The labeled samples were analyzed using an Agilent 1200 series nanoflow liquid chromatography system (Agilent Technologies) interfaced with 6530 Q-TOF mass spectrometer (Agilent Technologies) (Shi et al. 2013). A HPLC-Chip Cube MS Interface (Agilent Technologies) coupled with the columns PolySulfoethyl A SCX column (0.32×50 mm, 5 μm) and HPLC-Chip (Zorbax 300SB C18 enrichment column (0.3 mm×5 mm, 5 μm) and Zorbax 300SB C18 reversed-phase column (75 μm×50 mm, 3.5 μm) was used for two-dimensional analyses.

In the first dimension, 4 μL of the combined peptides mixture was loaded onto the SCX column and eluted stepwise by 8 μL of ammonium formate solutions in a series of concentrations—20, 40, 60, 80, 100, 300, 500, and 1000 mM. In the second dimension, the peptides were eluted from SCX column and trapped on the Zorbax 300SB C18 enrichment column using buffer A (5 % acetonitrile and 0.1 % formic acid in water) at a flow rate of 4 μL/min. The peptides which were trapped on the enrichment column were then eluted and separated by the Zorbax 300SB C18 reversed-phase column. The elution buffers used were Buffer B (0.1 % formic acid in water) and Buffer C (a nanoflow gradient of 5–80 % acetonitrile+0.1 % formic acid in water) at a flow rate of 300 nL/min. The total run time was 62 min for each round running. In all, nine runs were carried out for each sample due to the gradient elution. The effluent was analyzed by the MS. Survey scans were obtained from m/z 300 to 2000 at acquisition rate of 4 spectra per second, with up to two precursors selected for MS/MS analysis from m/z 50 to 2000 (Shi et al. 2013).

LC-MS/MS data analysis and interpretation

Protein identification and quantification were performed using Spectrum Mill software (Agilent Technologies). The MS/MS spectra acquired were searched against the database UniProtKB/Swiss-Prot with the following criteria: two maximum missed cleavages, 20 ppm precursor mass tolerance, 50 ppm product mass tolerance, methylmethanethiosulfate-labeled cysteine as the fixed modification, and iTRAQ modification of free amine in the amino terminus. For further statistical analysis, the following criteria were used: two or more identified unique peptides, protein MS/MS search score greater than 11, and peptide score greater than 6. Three independent experiments were carried out to calculate the standard deviation and confidence value. Those with more than 95 % confidence were selected for further analysis (Shi et al. 2013).

HPLC analysis of intracellular and extracellular metabolites

Metabolites in the filtered culture supernatant such as glucose, ethanol, glycerol, and acetate were quantified using an Agilent 1200 HPLC system (Singapore) equipped with a Rezex ROA-organic acid H+ (8 %) column (300×7.8 mm, Omega Scientific Phenomenex, Singapore). 5 mM H_2SO_4 was used as the mobile phase with a flow rate of 0.6 mL/min. The oven was set at 40 °C, and a refractive index detector was used. The samples were quantitated using standards.

ATP and NADPH assay

Intracellular ATP and NADPH were detected using ATP Colorimetric/Fluorometric Assay Kit and NADP/NADPH Quantitation Kit (BioVision, USA). Because they are labile during sampling, we used comparative ratio to indicate the changes between the engineered cells and the WT.

Results

In our previous work, it was shown that FFA production was enhanced in engineered strains △faa1△faa4 and △faa1△faa4 [Acot5s]. By comparing the fatty acids levels in the WT [Acot5s], △faa1, △faa1 [Acot5s], △faa4, △faa4 [Acot5s], △faa1△faa4, △faa1△faa4 [Acot5s], and WT strains, we observed a significant increase in extracellular FFA in the engineered strains △faa1△faa4 and △faa1△faa4 [Acot5s], while no significant differences were observed in the WT [Acot5s], △faa1, △faa1 [Acot5s], △faa4, and △faa4 [Acot5s]. The removal of genes FAA1 and FAA4 which encode for acyl-CoA synthetases led to the accumulation of a more than 4-fold extracellular fatty acid (326.97 μg/mL) as compared to the WT strain (76.5 μg/mL). The expression of cytosolic acyl-CoA thioesterase Acot5s in △faa1△faa4 [Acot5s] enhanced the total FFA production to about 500 μg/mL, which is more than 6-fold as compared to the WT, and 1.5-fold increase as compared to the strain △faa1△faa4. In addition, we also detected a high proportion of unsaturated extracellular fatty acids (UFA: C16:1 and C18:1) in △faa1△faa4 and △faa1△faa4 [Acot5s], whereas these could not be detected in the WT or the single deletion strains (Chen et al. 2014). Acyl-CoAs act as feedback inhibitors on fatty acid biosynthesis. The modification of acyl-CoA metabolism reduced the accumulation of acyl-CoAs, therefore relieving the inhibition and enhanced FFA production (Chen et al. 2014). The ability of S. cerevisiae to secrete FFA into medium also provided a driving force for fatty acid production, which is also seen in Runguphan and Keasling (2014). To obtain a global overview of the in vivo proteome changes in the engineered strains, a comparative proteomic analysis was performed.

iTRAQ analysis and protein identification

iTRAQ labeling was used to compare and analyze the in vivo protein changes between the WT, *△faa1 △faa4*, and *△faa1 △faa4* [*Acot5s*] strains. Proteins were extracted from samples harvested at 24 h, and labeled using the iTRAQ Reagent Multiplex Kit (AB SCIEX). Proteomic analysis was performed at least three times for statistical analysis. In total, over 500 proteins were detected. Among them, 82 proteins were found to be significantly altered in the engineered strains as compared to the WT as listed in Table S1 with their relative fold changes. Thirty proteins were found to be significantly altered in *△faa1 △faa4* [*Acot5s*] as compared to *△faa1 △faa4* as listed in Table 1.

Relative changes in protein expression between the WT and engineered strains

The identified proteins which were shown to have significant changes could be classified into different biological process categories according to their functions as annotated in the UniProtKB database (http://www.uniprot.org/) (Fig. 1). These functions included carbon metabolism, amino acid and nucleotide metabolism, protein biosynthesis, respiration, transportation, stress response, antioxidants, and others. The majority of proteins belonged to the category of carbon metabolism, which accounted for 37.80 % of all proteins (Fig. 1), showing that a major metabolic rearrangement occurred in the engineered cells as a result of the genetic modifications.

Among these proteins involved in carbon metabolism (Table S1), eight proteins (Glk1, Pyk1, Tpi1, Pgi1, Hxk1, Hxk2, Eno2, and Pdb1) which are involved in the glycolysis were upregulated in the strain *△faa4 △faa1*, whereas five proteins (Glk1, Hxk1, Gpm2, Eno2, and Pdb1) were upregulated in the strain *△faa4 △faa1* [*Acot5s*]. Enzyme Adh1 which is responsible for ethanol production showed upregulation in the strain *△faa4 △faa1*, whereas it remained unchanged in the strain *△faa4 △faa1* [*Acot5s*]. Four enzymes (Ald6, Ald4, Acs2, and Ach1) involved in acetate metabolism were upregulated in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains. Apart from the acetate biosynthetic process (Saint-Prix et al. 2004), Ald6 and Ald4 are also involved in NADPH regeneration (Grabowska and Chelstowska 2003). Acetyl-CoA synthetase 2 (Acs2) is involved in acetyl-CoA synthesis from acetate in the pyruvate-acetaldehyde-acetate pathway (Falcón et al. 2010). Ach1 is involved in shuttling mitochondrial acetyl-CoAs for cytosolic C2 provision (Chen et al. 2015) and acetate detoxification (Fleck and Brock 2009). The enzyme Gpp1 (Påhlman et al. 2001) which takes part in the glycerol biosynthetic process was downregulated in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains, whereas the enzyme Gut2 (Wang et al. 2001) involved in the glycerol catabolic process

was upregulated in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains. Moreover, two enzymes (Acc1 and Acb1) which participate in fatty acid metabolism, one enzyme (Icl1) involved in glyoxylate cycle (Schöler and Schüller 1993), and six enzymes (Cit1, Idh1, Idh2, Kgd1, Fum1, and Mdh1) involved in the TCA cycle were upregulated in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains. Four enzymes (Gnd1, Tkl1, Tal1, and Nqm1) involved in the pentose phosphate pathway were upregulated in the strain *△faa4 △faa1*, whereas two enzymes (Gnd1 and Tal1) were upregulated in the strain *△faa4 △faa1* [*Acot5s*]. For enzymes involved in carbohydrate biosynthesis, the major phosphoglucomutase isoform Pgm2 (Fu et al. 2000) responsible for hexose metabolism showed upregulation in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains. A previous study showed that protein abundance of Pmg2 also increased in response to various stresses (Hirata et al. 2003). For enzymes involved in the ergosterol biosynthetic process, Erg13 (Barker et al. 2003) was upregulated in *△faa4 △faa1*, whereas it was downregulated in *△faa4 △faa1* [*Acot5s*].

Three enzymes (Gas1, Mpg1, and Pmi40) involved in cell wall synthesis were downregulated in both *△faa4 △faa1* and *△faa4 △faa1* [*Acot5s*] strains. Six enzymes (Oye2, Atp1, Atp2, Atp3, Atp5, and Aac2) involved in respiration were upregulated in *△faa4 △faa1*, while five enzymes (Atp1, Atp2, Atp3, Atp5, and Aac2) were detected upregulated in *△faa4 △faa1* [*Acot5s*]. For proteins involved in the amino acid biosynthesis pathway, it was detected that eight enzymes (Arg1, Arg4, Sah1, Shm2, Lys1, Lys12, Lys21, and His4) were downregulated and four enzymes (Cys3, Ser3, Gdh1, and Uga1) were upregulated in *△faa4 △faa1*. Ten enzymes (Arg1, Arg4, Sah1, Ser3, Shm2, Lys1, Lys12, Lys21, Gdh1, and His4) were downregulated and one enzyme Cys3 was upregulated in *△faa4 △faa1* [*Acot5s*]. For proteins involved in the protein biosynthesis pathway, four enzymes (Pdi1, Ils1, Fpr3, and Stm1) were upregulated in the strain *△faa4 △faa1*, and three enzymes (Pdi1, Fpr3, and Rps2) were upregulated in the strain *△faa4 △faa1* [*Acot5s*].

For proteins involved in sugar, protein, and other ion transport process, six proteins (Hxt6, Pma1, Trx2, Por1, Chc1, and Grx1) were upregulated in the strain *△faa4 △faa1*. Among these proteins, all other five proteins except Chc1 showed similar increase in the strain *△faa4 △faa1* [*Acot5s*]. Twelve proteins (Gre2, Mcr1, Tps1, Tps2, Dcs1, Ynk1, Prb1, Glr1, Hsp26, Hsp42, Hsp104, and Ara1) involved in stress response showed increase in their expression levels in the strain *△faa4 △faa1*. Comparatively, six proteins (Gre2, Mcr1, Dcs1, Hsp26, Hsp30, and Hsp104) involved in stress response showed increase in the strain *△faa4 △faa1* [*Acot5s*].

The level of antioxidant proteins Ahp1 (Lee et al. 1999), Sod1 (Sturtz et al. 2001), and Sod2 (Saffi et al. 2006) were increased in the strain *△faa4 △faa1*, while only Sod1 was increased in the strain *△faa4 △faa1* [*Acot5s*]. Moreover, Rib4

Table 1 List of differentially expressed proteins in the strains *△faa1 △faa4* [*Acot5s*] as compared with the strain *△faa1 △faa4*
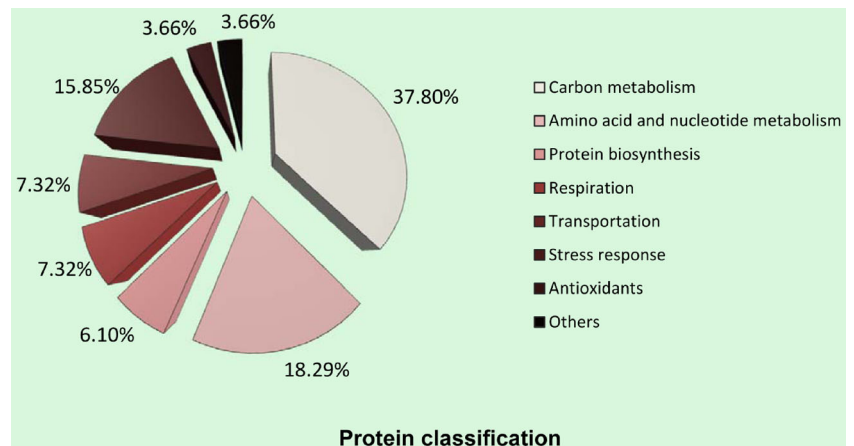
Pathway	Gene name	Protein name	Fold change[a]
Glycolysis	PKY1	Pyruvate kinase 1	0.73±0.021
	PGI1	Glucose-6-phosphate isomerase	0.66±0.040
	HXK2	Hexokinase-2	0.76±0.068
Pyruvate branchpoint	ADH1	Alcohol dehydrogenase 1	0.49±0.052
Acetate metabolism	ACH1	Acetyl-CoA hydrolase	1.90±0.340
Glycerol biosynthesis	GPP1	(DL)-glycerol-3-phosphatase 1	0.70±0.151
Glyoxylate cycle	ICL1	Isocitrate lyase	1.27±0.012
TCA cycle	KGD1	2-Oxoglutarate dehydrogenase, mitochondrial	1.19±0.049
	MDH1	Malate dehydrogenase, mitochondrial	1.31±0.053
Ergosterol biosynthesis	ERG13	Hydroxymethylglutaryl-CoA synthase	0.58±0.133
Amino acid biosynthesis	CYS3	Cystathionine gamma-lyase	1.49±0.261
	SER3	D-3-phosphoglycerate dehydrogenase 1	0.58±0.061
	LYS21	Homocitrate synthase, mitochondrial	0.77±0.078
	GDH1	NADP-specific glutamate dehydrogenase 1	0.37±0.017
	UGA1	4-Aminobutyrate aminotransferase	0.73±0.088
Cell wall synthesis	GAS1	1,3-Beta-glucanosyltransferase GAS1	0.82±0.051
	PMI40	Mannose-6-phosphate isomerase	0.74±0.076
Respiration	ATP1	ATP synthase subunit alpha, mitochondrial	1.54±0.351
	ATP3	ATP synthase subunit gamma, mitochondrial	1.22±0.119
	AAC2	ADP, ATP carrier protein 2	1.27±0.159
Stress response	TPS1	Trehalose-6-phosphate synthase	0.58±0.077
	TPS2	Trehalose-6-phosphate phosphatase	0.61±0.074
	GLR1	Glutathione reductase	0.81±0.08
	HSP42	Heat shock protein 42	0.55±0.132
	HSP104	Heat shock protein 104	0.76±0.07
	ARA1	D-arabinose dehydrogenase	0.76±0.111
Antioxidants	AHP1	Alkylhydroperoxide reductase	0.80±0.057
	SOD2	Superoxide dismutase [Mn], mitochondrial	0.75±0.102
Others	RIB4	6,7-Dimethyl-8-ribityllumazine synthase	0.38±0.045
	SAC1	Phosphoinositide phosphatase SAC1	1.15±0.127

[a] Data represented mean±S.D. based on three independent replicate experiments. Fold change refers to the average ratio of protein expression level in the strain *△faa1 △faa4* [*Acot5s*] over that in the strain *△faa1 △faa4*

involved in cofactor riboflavin biosynthesis (García-Ramírez et al. 1995), inositol-3-phosphate synthase (Ino1) (Shirra et al. 2001) involved in inositol synthesis, and phosphoinositide phosphatase Sac1 (Whitters et al. 1993) involved in the

Fig. 1 Functional classification of proteins identified. Proteins were classified into eight groups according to their functions annotated in the UniProtKB database (http://www.uniprot.org/). The percentage of proteins of each function group out of the whole proteins identified was exhibited

Protein classification

- Carbon metabolism — 37.80%
- Amino acid and nucleotide metabolism — 18.29%
- Protein biosynthesis — 6.10%
- Respiration — 7.32%
- Transportation — 7.32%
- Stress response — 15.85%
- Antioxidants — 3.66%
- Others — 3.66%

coordination of the activities of the secretory pathway showed upregulation in both strains.

Relative changes in protein expression between △faa4△faa1 [Acot5s] and △faa4△faa1

To investigate the influence of Acot5s expression on proteome changes, a direct comparison of protein changes between △faa4△faa1 [Acot5s] and △faa4△faa1 was conducted. As shown in Table 1, 10 proteins which play a role in carbon metabolism were found to be markedly changed in △faa1△faa4 [Acot5s] as compared to △faa1△faa4. Among these proteins, three enzymes (Pyk1, Pgi1, and Hxk2) involved in glycolysis, one enzyme (Adh1) involved in ethanol production, one enzyme (Gpp1) involved in the glycerol biosynthetic process, and one enzyme (Erg13) involved in the ergosterol biosynthesis pathway were downregulated in the strain △faa4△faa1 [Acot5s]. One enzyme (Ach1) that is involved in acetate metabolism, two enzymes (Kgd1 and Mdh1) involved in the TCA cycle, and one enzyme (Icl1) involved in glyoxylate cycle were upregulated.

Moreover, three proteins (Atp1, Atp3, and Aac2) involved in respiration and one phosphoinositide phosphatase Sac1 were upregulated in △faa4△faa1 [Acot5s]. Six proteins involved in stress response (Tps1, Tps2, Glr1, Hsp42, Hsp104, and Ara1) and two antioxidant proteins (Ahp1 and Sod2) were found downregulated. Two enzymes (Gas1 and Pmi40) involved in cell wall synthesis and one enzyme (Rib4) involved in cofactor biosynthesis were also downregulated in △faa4△faa1 [Acot5s]. Besides, five enzymes involved in amino acid biosynthesis were found differentially expressed.

Physiological and metabolite analysis of the engineered and WT strains

The WT and engineered strains were cultured in shake flask with YNB medium containing 20 g/L glucose as the carbon source. Growth curve and the concentration of several extracellular metabolites were studied. As shown in Fig. 2a, strain △faa1△faa4 had a defective growth and slower exponential growth rate, while strain △faa1△faa4 [Acot5s] displayed a higher growth rate as compared with the WT strain. This was also seen in our previous study (Chen et al. 2014). As showed in Fig. 2b, glucose consumption was delayed in the strain △faa1△faa4 as compared with the WT and △faa1△faa4 [Acot5s], the delayed glucose consumption in strain △faa1△faa4 was consistent with its slow growth rate. As for glycerol accumulation (Fig. 2c), △faa1△faa4 [Acot5s] had the lowest glycerol concentration, whereas the WT strain had the highest glycerol concentration. For acetate accumulation (Fig. 2d), △faa1△faa4 and △faa1△faa4 [Acot5s] had much lower final acetate concentration as compared with the WT strain. Regarding ethanol accumulation (Fig. 2e), the three

Fig. 2 Physiological features of the engineered and WT strains. The WT ▶ strain and engineered strains were cultured in YNBD medium. Cell growth (**a**), extracellular concentration of glucose (**b**), glycerol (**c**), acetate (**d**), and ethanol (**e**) were measured at different time points as indicated in method. Data are the mean values of three independent experiments. The *error bar* indicates the S.D.

strains had similar ethanol concentration curve. The strain △faa1△faa4 [Acot5s] gradually accumulated a lower level of ethanol after 24 h than the WT and △faa1△faa4 strains. In addition, intracellular ATP and NADPH levels of three strains were examined. As expected, the strains △faa1△faa4 and △faa1△faa4 [Acot5s] had higher ATP and NADPH levels as compared to the WT strain in both exponential phase and stationary phase (Figs. 3 and 4).

Discussion

In our previous study, it was shown that FFA production was enhanced by disrupting both acyl-CoA synthetases Faa1 and Faa4, and expressing acyl-CoA thioesterase Acot5s in *S. cerevisiae*. This modification restrained FFA activation, relieved the feedback inhibition of acyl-CoAs on fatty acid synthesis, and directed carbon flux toward FFA accumulation (Chen et al. 2014). However, how the cells sensed the changes in fatty acid metabolism and responded accordingly was unknown. Therefore, we carried out proteomic analysis in this study, and mapped the changes in proteins in relation to the biochemical pathways to give a global overview of the pathway regulations (Figs. 5 and 6).

Comparative proteome analysis of the engineered strains as compared to the WT

There were many changes in enzymes involved in crucial biological pathways, due to the double deletion of genes *FAA1* and *FAA4* and expression of *Acot5s*. As shown in Table S1, several enzymes involved in glycolysis, a fundamental pathway in glucose metabolism to provide energy in yeast (Bruce Alberts and Lewis 2002), were upregulated in both engineered strains. The average increment of these enzymes was 30.75 % in △faa1△faa4 and 30.2 % in △faa1△faa4 [Acot5s]. In the glycerol metabolism pathway, the abundance of the enzyme Gpp1 (Påhlman et al. 2001), which is involved in the glycerol biosynthetic process, was found to decrease to 79 and 53 % in the strains △faa1△faa4 and △faa1△faa4 [Acot5s], respectively, as compared with the WT strain. The relative abundance of the enzyme Gut2 (Wang et al. 2001), which is involved in the glycerol catabolic process, increased more than 30 % in both engineered strains. This was consistent with the data that both engineered strains accumulated less glycerol than the WT strain (Fig. 4c). In addition, decrease
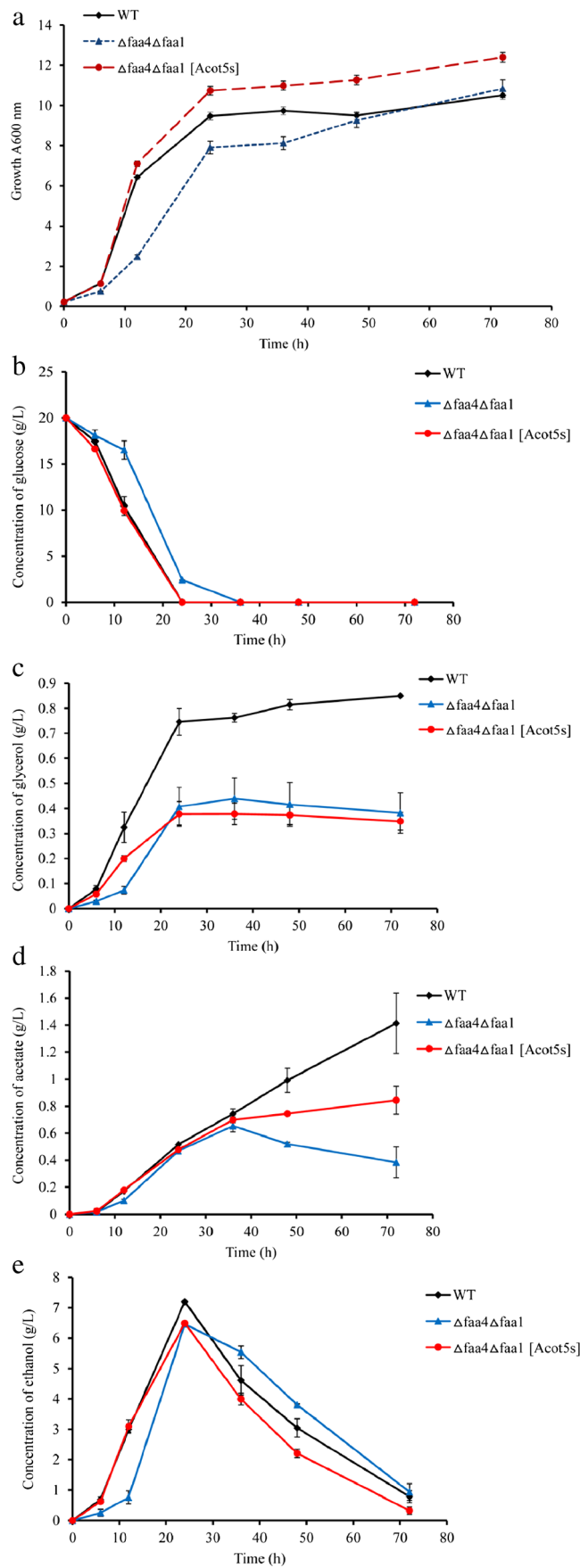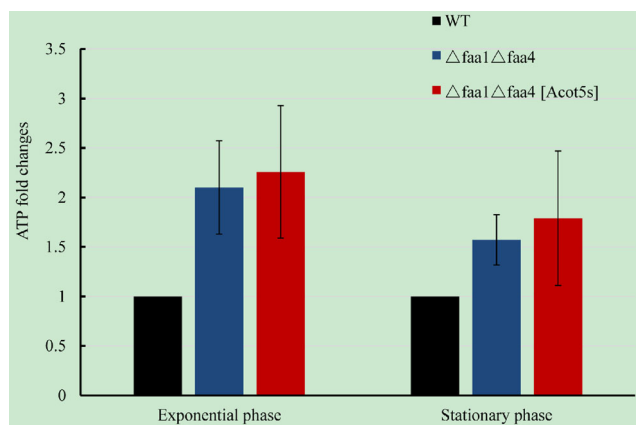
Fig. 3 ATP level in the engineered strains as compared with the WT strain. The amount of ATP in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 8 and 24 h. The *error bar* indicates the S.D.

in intracellular glycerol levels were also seen in both engineered strains in our previous metabolite profiling data (Chen et al. 2014). The decrease in glycerol synthesis probably provided extra pyruvate for other pathways. In the pyruvate-ethanol pathway, the relative abundance of protein Adh1, which is the main alcohol dehydrogenase that catalyzes the conversion of acetaldehyde to ethanol (Leskovac et al. 2002), increased 2-fold in the strain *Δfaa1Δfaa4*, while it remained unchanged in the strain *Δfaa1Δfaa4 [Acot5s]*. Because several proteins in the pyruvate-acetaldehyde-acetate pathway were upregulated in *Δfaa1Δfaa4 [Acot5s]*, it is not a surprise that the strain *Δfaa1Δfaa4 [Acot5s]* had lower concentration of ethanol than the WT and *Δfaa1Δfaa4* after 24 h (Fig. 2e). However, we did not find significant increase of ethanol concentration in the strain *Δfaa1Δfaa4*. This may be due to it had slower growth rate and lower biomass accumulation than the WT strain, and several proteins in its pyruvate-acetaldehyde-acetate pathway were also upregulated. There were three enzymes (Erg10, Erg13, and Erg20) detected in
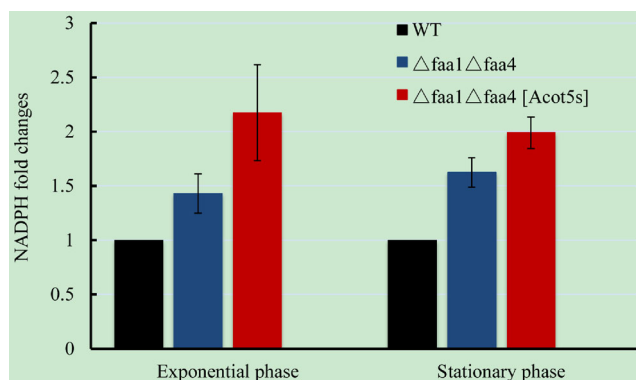

Fig. 4 NADPH level in the engineered strains as compared with the WT strain. The amount of NADPH in the WT strain was set as 1. The values are the means from three experiments examining cell extracts at 8 and 24 h. The *error bar* indicates the S.D.

the ergosterol biosynthesis pathway. Erg13 were upregulated to 112 % in *Δfaa4Δfaa1* and downregulated to 64 % in *Δfaa4Δfaa1 [Acot5s]* as compared to the WT. There were no significant changes in the level of Erg10 and Erg20 (data not shown). This might indicate that there was decreased carbon flux toward ergosterol production in the strain *Δfaa4Δfaa1 [Acot5s]*. Further experiment to estimate the regulation of the ergosterol pathway in *Δfaa4Δfaa1* would be required.

Besides the abovementioned carbon metabolic regulation, multiple levels of regulation in fatty acid metabolism of *S. cerevisiae* exist. Fatty acid synthesis is a highly energy-demanding process, which requires large amounts of acetyl-CoA, ATP, and NADPH (Tehlivets et al. 2007). The supply of these compounds has significant effect on fatty acid synthesis. Acetyl-CoA in *S. cerevisiae* can be produced from both the mitochondrial pyruvate dehydrogenase pathway and the cytosolic pyruvate-acetaldehyde-acetate pathway (Chen et al. 2012; Pronk et al. 1996). As shown in Table S1, enzyme Pdb1 (Miran et al. 1993), which is part of the pyruvate dehydrogenase complex and involved in catalyzing the overall conversion of pyruvate to acetyl-CoA in the mitochondria, was upregulated in both strains. The levels of several enzymes in the pyruvate-acetaldehyde-acetate pathway were also increased. Cytosolic aldehyde dehydrogenase (Ald6) which plays a major role in acetate formation and a compensative mitochondrial aldehyde dehydrogenase (Ald4) (Saint-Prix et al. 2004) were upregulated in both engineered strains. Acetyl-CoA synthetase 2 (Acs2) (Carman et al. 2008; Van Den Berg and Steensma 1995), which generates acetyl-CoA by direct activation of acetate in the cytoplasm, was also upregulated by about 15 % in both strains. This was consistent with the observation that both engineered strains accumulated less acetate than the WT strain (Fig.2d). In addition, the enzyme Icl1 (Schöler and Schüller 1993), which catalyzes the formation of succinate and glyoxylate from isocitrate, a key step in the glyoxylate cycle, was also upregulated. Glyoxylate cycle is one known route for transporting acetyl-CoA from the peroxisomes/cytosol into the cytosol/mitochondria through converting acetyl-CoA into glyoxylate cycle intermediates (van Roermund et al. 1995). The upregulation of Icl1 in the glyoxylate cycle implied there may be increased need for acetyl-CoA transportation. Furthermore, the upregulation of Ach1 may imply the same increased need, since mitochondrial Ach1 can transport acetyl-CoA produced in the mitochondria to the cytosol via conversion of acetyl-CoA into acetate, which can cross the mitochondrial membrane freely (Chen et al. 2015). Although we did not observe significant increase in cellular acetyl-CoA level in the engineered strains (data not shown), the significant increase of FFA production implied that there should be increased supply of acetyl-CoA.

ATP can be generated through oxidation of glucose in glycolysis, the TCA cycle, and oxidative phosphorylation. The amount of energy released by oxidative phosphorylation is

Fig. 5 Changes of metabolic pathways in the strain △*faa1* △*faa4* as compared with the WT. The primary pathways are described according to the changes in protein expression as listed in Table S1. *Red boxes* and *red text* indicate upregulated pathways and proteins. *Blue boxes* and *blue text* indicate downregulated pathways and proteins
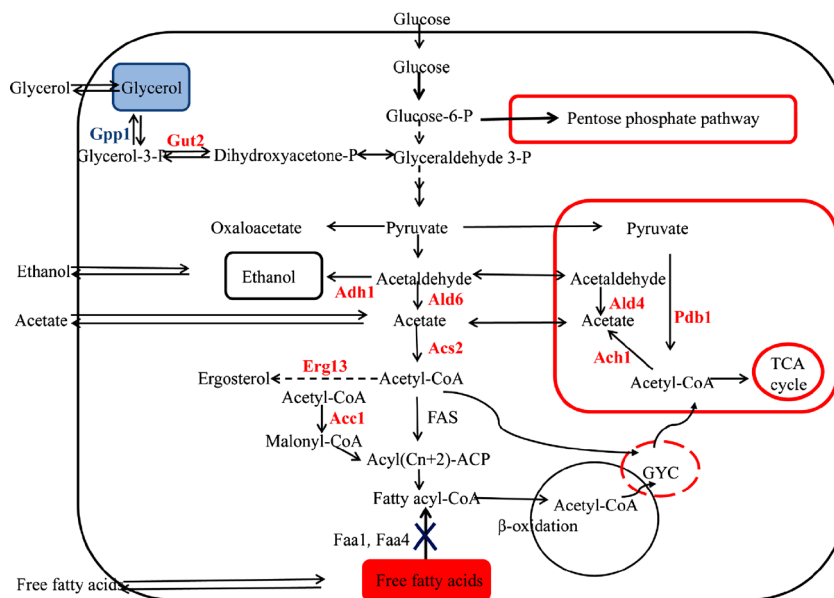
much higher as compared to glycolysis (Rich 2003). In addition to the upregulation of enzymes in glycolysis, six enzymes involved in the TCA cycle, and several subunits of ATP synthase (Reinders et al. 2007) in aerobic respiration were observed to be significantly upregulated in both engineered strains. In the strain △*faa1* △*faa4*, enzymes in the TCA cycle were upregulated from 1.13- to 1.59-fold, and subunits of ATP synthase were upregulated from 1.18- to 1.24-fold as compared to the WT strain. In the strain △*faa1* △*faa4* [*Acot5s*], enzymes in the TCA cycle were upregulated from 1.21-to1.80-fold, and subunits of ATP synthase were upregulated from 1.26- to 1.62-fold as compared to the WT strain. Besides, the major ADP/ATP carrier of the mitochondrial inner

membrane Aac2 was also upregulated in both engineered strains. Aac2 imports cytosolic ADP into the mitochondria, where it can be phosphorylated by ATP synthase, and exports ATP into the cytosol (Klingenberg 2008; Ruprecht et al. 2014). So, the upregulation of Aac2 was consistent with the upregulation of ATP synthase. The analysis of the ATP level in three strains demonstrated there were higher levels of ATP in two engineered strains as compared to the WT, which further demonstrated the upregulation of ATP synthesis.

NADPH can be provided by several sources such as malic enzyme, the pentose phosphate pathway, NADPH isocitrate dedydrogenase, NAD kinase (Tehlivets et al. 2007), and the aldehyde dehydrogenase family (Grabowska and

Fig. 6 Changes of metabolic pathways in the strain △*faa1* △*faa4* [*Acot5s*] as compared with the WT. The primary pathways are described according to the changes in protein expression as listed in Table S1. *Red boxes* and *red text* indicate upregulated pathways and proteins. *Blue boxes* and *blue text* indicate downregulated pathways and proteins

Fig. 7 Changes of metabolic pathways in the strain △*faa1* △*faa4* [*Acot5s*] as compared with △*faa1* △*faa4*. The primary pathways are described according to the changes in protein expression as listed in Table 1. *Red boxes* and *red text* indicate upregulated pathways and proteins. *Blue boxes* and *blue text* indicate downregulated pathways and proteins
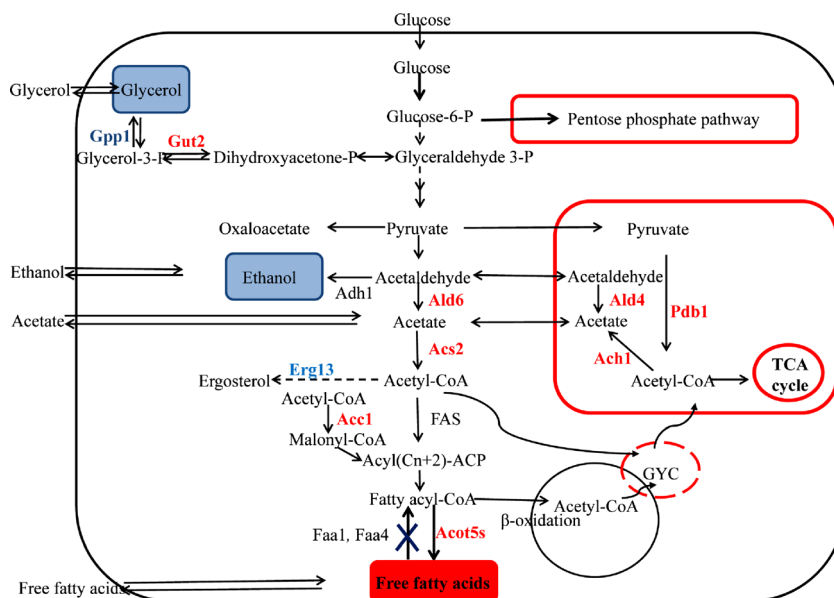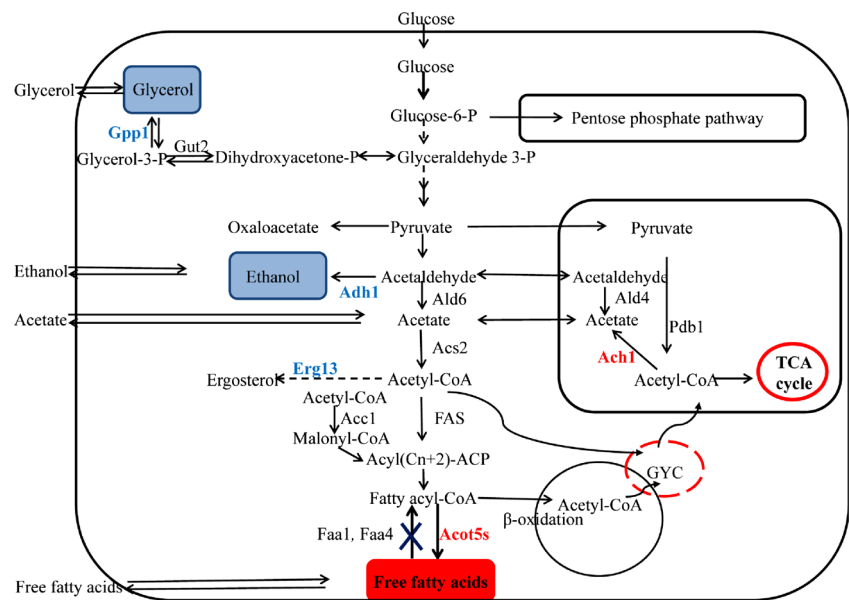

Chelstowska 2003; Minard and McAlister-Henn 2005). In the present analysis, we found that several enzymes involved in NADPH generation were upregulated. For example, the level of Gnd1 (6-phosphogluconate dehydrogenase), which catalyzes the NADPH generation step in the pentose phosphate pathway, increased by about 55 % in both strains. Ald6 and Ald4, which utilize NADP+ as the preferred coenzyme when performing the conversion of acetaldehyde to acetate and play important roles in NADPH regeneration (Grabowska and Chelstowska 2003), were upregulated by 25 and 26 % in △*faa1* △*faa4*, 15 and 43 % in △*faa1* △*faa4* [*Acot5s*]. The increased intracellular NADPH level in both engineered strains further proved there was increased NADPH synthesis in both engineered strains.

Furthermore, the enzymes involved in fatty acid synthesis were also altered, although the results were different from the transcriptional level analysis (Chen et al. 2014); that is, the expression of genes related to fatty acid synthesis which are repressed by fatty acyl-CoAs were increased significantly in the engineered strains △*faa1* △*faa4* and △*faa1* △*faa4* [*Acot5s*], especially *OLE1* which encodes a △−9 fatty acid desaturase. However, the protein Acc1 was increased by only about 15 %, while the RT-PCR result showed several fold increase. The increase in Acc1 protein level was smaller than its increase in gene expression level. There was no observed significant increase in the level of Fas1 and Fas2. △−9 fatty acid desaturase was not detected, which may be due to its location in the endoplasmic reticulum membrane (Kim et al. 2006). Also, regulation of these proteins is complex and occur at multiple levels, including histone acetylation, mRNA stability, protein stability, and coordination with other genes expression (Tehlivets et al. 2007). The increase in mRNA level with no subsequent increase in protein expression level is

understandable and also supported by other studies (Griffin et al. 2002; Ideker et al. 2001). Although Acc1 affects fatty acid synthesis directly and overexpression of Acc1 could enhance fatty acid synthesis (Davis et al. 2000; Lennen et al. 2010; Ruenwai et al. 2009), overexpression of Acc1 in eukaryotic organism generally results in small improvement of fatty acid production (Li et al. 2014; Runguphan and Keasling 2014). Further study is needed to explore the role of upregulation of Acc1 in the increased fatty acid production. Moreover, the level of acyl-CoA binding protein Acb1, which facilitates the cellular transportation of acyl-CoA and lessens the inhibition effect of acyl-CoA on fatty acid synthesis (Rasmussen et al. 1993; Vock et al. 2010), was increased 2.45-fold and 2.43-fold, respectively, in △*faa1* △*faa4* and △*faa1* △*faa4* [*Acot5s*].

Several proteins involved in transportation were found to be upregulated in both engineered strains, especially the high-affinity hexose transporter Hxt6, which plays an important role in glucose uptake and utilization (Reifenberger et al. 1995). It was upregulated more than 3-fold in both strains. This was consistent with the upregulation of glycolysis in both strains. Previous study showed that overproduction of fatty acids in *E. coli* resulted in the change of membrane properties because of fatty acid intercalation in the membrane, fatty acid accumulation in the periplasm, and alteration of membrane lipids; these changes then led to membrane stress and compromised membrane integrity (Lennen et al. 2011). In our engineered strains, the downregulation of enzymes (Gas1, Mpg1, and Pmi40) involved in cell wall synthesis (Payton et al. 1991; Tomishige et al. 2003; Yoda et al. 2000) and upregulation of enzyme Sac1 (phosphoinositide phosphatase) involved in the secretory

pathway (Whitters et al. 1993) should also be the response of fatty acid overproduction, such regulation may facilitate FFA to be secreted outside the cells.

In cells, amino acid biosynthesis pathways are not only an essential process for protein synthesis, they are also critical for yeast cells to achieve coordinated regulation between nitrogen source utilization and nucleotide biosynthetic pathways (Ljungdahl and Daignan-Fornier 2012). In our observation, many enzymes involved in amino acid biosynthesis (Table S1) were differentially expressed in two engineered strains as compared to the WT. Among these enzymes, Shm2 (McNeil et al. 1994) is the major enzyme responsible for converting serine to glycine. Lys1, Lys12, and Lys21 (Mark Zabriskie and Jackson 2000) are responsible for L-lysine biosynthesis. The downregulation of these enzymes was consistent with the observed decrease in L-lysine and glycine levels in previous metabolic profiling result (Fig. 6 in (Chen et al. 2014)). For other enzymes involved in amino acid synthesis, there were no significant changes detected in the relevant amino acids levels in our previous study. Since cells have a natural ability to reduce biosynthetic cost to optimize growth and reproduction, the regulation of amino acid, protein, and nucleotide biosynthesis should be under the need of metabolic cost optimization (Barton et al. 2010).

Also, DNA microarray experiment showed that environmental stresses, including temperature shock and oxidative stress, greatly stimulate upregulation of the transcription of stress-responsive genes in yeast cells (Gasch et al. 2000). In our study, we observed that more enzymes involved in stress response and antioxidants were upregulated in the strain △faa1△faa4 as compared to the strain △faa1△faa4 [Acot5s]. For example, D-arabinose dehydrogenase Ara1 (Hu et al. 2013; van Bergen et al. 2006), which was re-annotated as α, β-dicarbonyl reductase and found involved in reducing a variety of toxic metabolic by-products such as methylglyoxal, diacetyl, and pentanedione produced during stress, was only upregulated in △faa1△faa4. This was consistent with the growth defect of the strain △faa1△faa4 and recovered growth of the strain △faa1△faa4 [Acot5s] (Fig. 2a), which indicated the stresses caused by removing Faa1 and Faa4 were relieved in a certain degree by introducing Acot5s.

The impact of Acot5s expression on proteome changes

As stated above, the growth phenotype of the strain △faa4△faa1 is more severe than the strain △faa4△faa1 [Acot5s]; thus, it is not surprising that there are more changes about proteins involved in central metabolism in △faa4△faa1 as opposed to △faa4△faa1 [Acot5s]. We wonder how the △faa4△faa1 [Acot5s] cells responded to the driving force provided by Acot5s and had higher FFA production and growth rate compared to △faa4△faa1 and WT strains. Through comparatively analyzing protein expression levels between

△faa4△faa1 [Acot5s] and △faa4△faa1, the affected proteins that take part in crucial metabolic pathways were mapped in Fig. 7. Among them, the level of alcohol dehydrogenase Adh1, which is involved in ethanol production, decreased to 49 % in △faa4△faa1 [Acot5s] as compared to △faa4△faa1. The level of enzyme Gpp1 involved in the glycerol biosynthetic process was downregulated to 70 % in △faa4△faa1 [Acot5s] as compared to △faa4△faa1. This indicated that the added driving force provided by Acot5s made cells a direct part of their carbon flux from ethanol and glycerol production to acetyl-CoA synthesis. Although we did not observe additional increase in protein level involved in the pyruvate-acetaldehyde-acetate pathway, we did found decrease in ethanol and glycerol production and increase of FFA production in the strain △faa4△faa1 [Acot5s]. In addition, Erg13 involved in the ergosterol biosynthesis pathway were downregulated to 58 % of that in △faa4△faa1, which may imply decreased carbon flux toward ergosterol.

Protein Ach1, which is involved in acetyl-CoA transportation and acetate detoxification (Chen et al. 2015), was upregulated 1.9-fold in △faa4△faa1 [Acot5s] as compared to △faa4△faa1. In addition, the reported study (Chen et al. 2015) also showed that overexpression of ACH1 in the △ach1 strain not only rescued growth on glucose but also led to slightly faster growth than the △ach1 strain. The higher expression level of Ach1 may explain the higher growth rate of △faa4△faa1 [Acot5s]. Furthermore, the downregulation of stress response and antioxidants proteins also showed the △faa4△faa1 [Acot5s] cells adapted to the new balanced state built by Acot5s. Moreover, the upregulation of protein Kgd1 and Mdh1 (1.19- and 1.31-fold) involved in the TCA cycle, and Atp1, Atp3, and Aac2 (1.54-, 1.22- and 1.27-fold) involved in respiration were consistent with the higher ATP level.

The direct effect of removing acyl-CoA synthetases Faa1 and Faa4 was restraining FFA activation, and led to FFA secretion into the culture medium as demonstrated before (Scharnewski et al. 2008). Although the mechanism of fatty acid secretion has not been unraveled completely yet, it was reported that the fatty acid transport could be driven by the concentration gradient between extracellular and intracellular fatty acid and need the activities of fatty acyl-CoA synthetase (Faa1 and Faa4) and fatty acid transport protein 1 (Fat1). The deletion of FAA1 and FAA4 increases the concentration of FFA in the cytosol which leads to the secretion of FFA (Scharnewski et al. 2008), it also decreases the concentration of intracellular acyl-CoA which results in changes in intracellular signaling to fatty acid-responsive genes (Black and DiRusso 2007; Færgeman et al. 2001). In our study, the expression of thioesterase reinforced the effect; thus, the modification provided a higher driving force toward higher fatty acid production. Compared with other recent works (Leber et al. 2015; Li et al. 2014; Runguphan and Keasling 2014),

besides using different background strains, the main difference between these studies is the driving force, which is provided by different thioesterases (Li et al. 2014; Runguphan and Keasling 2014) or lipid body forming enzymes with lipase (Leber et al. 2015). Our study showed that Acot5s is a pretty efficient enzyme to transform acyl-CoA to FFA. The expression of Acot5s affected the regulation of proteins involved in crucial metabolic pathways, which led to a major metabolic rearrangement while not harming growth.

In summary, to gain insights into the underlying metabolic regulation associated with the genetic modification in the engineered strains, we set out to investigate the proteomic profile changes between WT and engineered strains. It was found that the disruption of genes *FAA1* and *FAA4* and expression of *Acot5s* in *S. cerevisiae* contributed to elevated carbon flux toward FFA accumulation. It was identified that proteins involved in glycolysis, the pentose phosphate pathway, TCA cycle, fatty acid synthesis, and ATP synthesis and transportation were upregulated, while proteins involved in glycerol synthesis, pyruvate-ethanol synthesis, ergosterol synthesis, and cell wall synthesis were downregulated in the strain *△faa1 △faa4 [Acot5s]*. Our detected changes in metabolites supported the finding regarding the changes in protein expression in the different metabolic pathways. Previous study demonstrated that the genetic modification relieved the feedback inhibition of fatty acyl-CoAs (Chen et al. 2014). The global metabolic regulation found in this study indicated that the cells upregulated certain pathways to facilitate fatty acid synthesis and substrates supply, and efficiently utilize the carbon flux by downregulating some pathways and channeling the carbon flux toward FFA accumulation. This information is of great help to unravel the mechanism for the increased production of FFA in the engineered strain. It also shows the great potential of the engineered strain in the metabolic adjustment, and it will certainly aid future work in rational engineering of the yeast system for biofuel production. For example, the flux toward alcohol biosynthesis could be further decreased in the engineered strain, and enzymes in the pyruvate-acetaldehyde-acetate pathway or Ach1p could be overexpressed to reinforce the carbon flux toward cytosolic acetyl-CoA supply. Several genes in β-oxidation could be deleted to diminish cellular fatty acid degradation. More importantly, the driving force could be enhanced to further promote FFA production.

Compliance with ethical standards

Conflict of interest Liwei Chen declares that she has no conflict of interest. Jaslyn Jie Lin Lee declares that she has no conflict of interest. Jianhua Zhang declares that he has no conflict of interest. Wei Ning Chen declares that he has no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

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