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Central Metabolic Responses to the Overproduction of Fatty Acids in *Escherichia coli* Based on ^{13}C -Metabolic Flux Analysis

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Abstract

We engineered a fatty acid overproducing *E. coli* strain through overexpressing *tesA* (“pull”) and *fadR* (“push”) and knocking out *fadE* (“block”). This “pull-push-block” strategy yielded 0.17 gram of fatty acids (C₁₂–C₁₈) per gram of glucose (equivalent to 48% of the maximum theoretical yield) in batch cultures during the exponential growth phase under aerobic conditions. Metabolic fluxes were determined for the engineered *E. coli* and its control strain using tracer ([1,2- ^{13}C]glucose) experiments and ^{13}C -metabolic flux analysis. Cofactor (NADPH) and energy (ATP) balances were also investigated for both strains based on estimated fluxes. Compared to the control strain, fatty acid overproduction led to significant metabolic responses in the central metabolism: 1) Acetic acid secretion flux decreased 10-fold; 2) Pentose phosphate pathway and Entner–Doudoroff pathway fluxes increased 1.5-fold and 2.0-fold, respectively; 3) Biomass synthesis flux was reduced 1.9-fold; 4) Anaplerotic phosphoenolpyruvate carboxylation flux decreased 1.7-fold; 5) Transhydrogenation flux converting NADH to NADPH increased by 1.7-fold. Real-time quantitative RT-PCR analysis revealed the engineered strain increased the transcription levels of *pntA* (encoding the membrane-bound transhydrogenase) by 2.1-fold and *udhA* (encoding the soluble transhydrogenase) by 1.4-fold, which is in agreement with the increased transhydrogenation flux. Cofactor and energy balances analyses showed that the fatty acid overproducing *E. coli* required significantly higher cellular maintenance energy than the control strain. We discussed the guidelines to future strain development and process improvements for fatty acid production in *E. coli*.

Graphical abstract

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The authors engineered a fatty acid producing *E. coli* that produced fatty acids with 48% of the theoretical yield. To reveal the metabolic bottlenecks, the authors investigated its central metabolism using ^{13}C -MFA and transcription analysis. They found that the native transhydrogenases were flexibly regulated to balance cofactors for fatty acid production, whereas overproducing fatty acids was limited by energy supply due to high maintenance energy caused by cell membrane stress. New metabolic engineering strategies were discussed to further enhance fatty acid production.

Keywords

isotope labeling; mass spectrometry; cofactor balance; energy balance; maintenance energy; metabolic engineering

Introduction

Fatty acids are the precursors to produce transportation fuels and industrial chemicals including surfactants, solvents and lubricants. Fatty acids are conventionally derived from plant oils and animal fats, which causes competition with food supply and environmental concerns. Alternative strategies have recently attracted interest in the production of fatty acids from abundant and inexpensive renewable resources through microbial fermentations (Ranganathan et al., 2012; Steen et al., 2010; Stephanopoulos, 2007).

E. coli can serve as an excellent host for fatty acids production due to its fast growth, simple nutrient requirements, well understood metabolic behavior and available genetic tools. However, fatty acid metabolism in *E. coli* is tightly regulated (Steen et al., 2010). Fatty acids synthesized in the wild-type *E. coli* are mainly used to form lipids for cell membrane constitution (Fujita et al., 2007; Magnuson et al., 1993), and only a small amount of free fatty acids are detectable under normal conditions (Steen et al., 2010). Fig. 1 shows the pathways of fatty acid metabolism in *E. coli*. The synthesis of saturated fatty acid starts with the conversion of acetyl-CoA into malonyl-CoA catalyzed by ATP-dependent acetyl-CoA carboxylase (AccABCD) and the transesterification of malonyl-CoA into an acyl carrier protein (ACP) catalyzed by malonyl-CoA ACP transacylase (FabD), and then followed by

cyclic chain elongation. The synthesis of unsaturated fatty acids starts with 3-hydroxydecanoyl-ACP, which is dehydrated by FabA (introducing a double bond to the fatty acid chain) before undergoing unsaturated fatty acid elongation (Feng and Cronan, 2009).

Acyl-ACP thioesterase (TesA) catalyzes the hydrolysis of fatty acyl-ACPs, “pulling” the carbon flux to fatty acid production (Steen et al., 2010; Zhang et al., 2011). Fatty acids can be degraded via the β -oxidation pathway (Fujita et al., 2007). The β -oxidation involves the activation of fatty acids to fatty acyl-CoAs catalyzed by FadD, followed by cleavage steps in the β -oxidation cycle to yield acetyl-CoA ultimately. The key step in the β -oxidation cycle is the oxidation of acyl-CoA to 2-enoyl-CoA, catalyzed by acyl-CoA dehydrogenase (FadE) (Campbell and Cronan Jr., 2002). “Blocking” the β -oxidation pathway by knocking out *fadD* or *fadE* can improve fatty acid accumulation (Lennen et al., 2011; Lu et al., 2008; Steen et al., 2010). Moreover, the fatty acid metabolism is regulated by the global transcription factor *fadR*. FadR binds to the promoters of several genes in the fatty acid metabolic pathways and controls their expression (Campbell and Cronan Jr., 2002; Magnuson et al., 1993). Specifically, FadR acts as the activator of *fabA* and *fabB* genes to “push” fatty acid production, and functions as the repressor of the *fad* regulon to “block” fatty acid degradation. The “pull-push-block” based metabolic engineering strategies (Fig. 1) have been reported to produce fatty acids at the level of less than $0.25 \text{ g} \cdot \text{g} \text{ glucose}^{-1}$ ($< 70\%$ of the maximum theoretical yield) in *E. coli* (Lennen et al., 2010; Li et al., 2012; Liu et al., 2010; Lu et al., 2008; Steen et al., 2010; Zhang et al., 2011; Zhang et al., 2012b; Xu et al., 2013).

Although researchers have created various fatty acid producing strains, most studies target engineering genes in the end fatty acid metabolic pathways. Little attention has been given to the central metabolism genetic interventions for improving fatty acid overproduction until recently. Li et al. (2012) engineered an *E. coli* strain with deleted acetate production pathway for fatty acid production but no improvement of fatty acid yield was achieved. Fatty acid production places high demands on precursor (acetyl-CoA), reducing power (NADPH) and energy (ATP) molecules ($8\text{Acetyl-CoA} + 14\text{NADPH} + 7\text{ATP} \rightarrow \text{Palmitate (C16:0 fatty acid)}$). Since these molecules are mainly derived in the central metabolism, it is important to investigate the behavior of the central metabolism as fatty acid overproduction in *E. coli* are likely to be challenged in balancing these factors (Lennen and Pfleger, 2012). To reveal the metabolic bottlenecks, we used the powerful ^{13}C -metabolic flux analysis (^{13}C -MFA) tool (Antoniewicz et al., 2007a; Antoniewicz et al., 2007b; Christensen et al., 2002; Stephanopoulos, 1999; Suthers et al., 2007; Young et al., 2008) to characterize the central metabolism of *E. coli* in response to fatty acid production. Specifically, we engineered a fatty acid producing *E. coli* strain through overexpressing *tesA* and *fadR* genes in a *fadE* knockout strain of *E. coli* DH1. The genetic strategy was modified from that in the recent report (Zhang et al., 2012b), which introduced *tesA* and *fadR* genes in separate plasmids into the host *E. coli* strain and produced fatty acids at a high yield. In this study, we integrated the *tesA* and *fadR* genes into a single plasmid so that only one type of antibiotic was required in the cultures and thus the antibiotic toxicity was minimized. We performed tracer ($[1,2-^{13}\text{C}]\text{glucose}$) experiments and ^{13}C -MFA for both the engineered strain and the control strain that contained an empty plasmid in the same host *E. coli* strain. We also evaluated the cofactor balance and energy status of both strains. Transcription levels of genes at the key nodes in central metabolic pathways were measured using real time quantitative RT-PCR

(qRT-PCR) for additional information. This study aims to provide insights into the metabolism of *E. coli* overproducing fatty acids and guide strain engineering for further improvement of fatty acid production.

Materials and Methods

Plasmid and Strain Construction

The plasmid pA58c-TR (Supplementary Fig. 1) was constructed from pE8a-fadR and pKS1 using a modified Golden Gate DNA assembly method (Steen et al., 2010; Zhang et al., 2012a; Zhang et al., 2012b) as described in Supplementary Materials and Methods. The constructed plasmid pA58c-TR containing the *tesA* and *fadR* genes was then transformed into a *fadE* knockout strain of *E. coli* DH1 (endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17(r_K⁻ m_K⁺) λ⁻) using calcium chloride (Sambrook and Russell, 2001), resulting in *E. coli* DH1 *fadE*/pA58C-TR (the engineered fatty acid producing strain). The control strains were constructed by transforming the vector pACYCDuet-1, which has the same backbone as pA58C-TR (pA15 ori, Cm^R), into the wild-type *E. coli* DH1 and the *E. coli* DH1 *fadE* strains using the above method, resulting in *E. coli* DH1/pACYC-Duet-1 and *E. coli* DH1 *fadE*/pACYC-Duet-1. We compared the growth and ¹³C labeling patterns of the proteinogenic amino acids of two control strains and found no differences under our cultivation conditions (data are not shown). We then used *E. coli* DH1 *fadE*/pACYC-Duet-1 as the control strain in the rest of the study.

Bacterial Cultivation With [1,2-¹³C]glucose

M9 MOPS minimal medium (Supplementary Materials and Methods) with [1,2-¹³C]glucose was used in the ¹³C labeling experiments. The first pre-culture inoculated from the glycerol stock was grown for 12 h in 5 mL LB medium. The cells were harvested by centrifugation at 8,000 × g and 4°C for 2 min, washed and used to inoculate the second pre-culture of 5 ml of minimal medium containing [1,2-¹³C]glucose. Cells from the second pre-culture were harvested, washed and used to inoculate the main culture. The main cultures were carried out in duplicate in 250 mL baffled shake flasks (Kimax, Fisher Scientific) containing 25 mL of minimal medium with [1,2-¹³C]glucose on a rotary shaker (Big Bill Orbital Shakers, Thermolyne, Thermo Scientific) at 225 rpm. The inoculation fraction was 1% (v/v) in each step. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was supplemented at the final concentration of 0.1 mM to induce *tesA* gene in 13 hours incubation. The *fadR* gene is under the control of pBAD promoter (Supplementary Fig. 1). Since the previous study (Zhang et al., 2012b) using the same promoter showed that the *fadR* expression could be induced by 7.5-fold in glucose medium, we did not supplement arabinose for pBAD promoter induction in the cultures. Biomass samples were taken at three time points during the exponential growth phase for isotopomer distribution analyses of proteinogenic amino acids. All cultivations were carried out at 37 °C.

The analyses of cell growth, extracellular metabolites and fatty acids are shown in Supplementary Materials and Methods.

Analysis of Mass Isotopomer Distributions of Proteinogenic Amino Acids

GC-MS analyses of proteinogenic amino acids were previously described (Feng et al., 2010). Briefly, the cells were washed twice with 0.9% NaCl and hydrolyzed in 6 mol·l⁻¹ HCl at 100 °C for 24 h. The resulting proteinogenic amino acids were derivatized with *N*-Methyl-*N*-[tert-butyldimethyl-silyl] trifluoroacetimide (Sigma-Aldrich) in tetrahydrofuran (Sigma-Aldrich) at 70°C for 1 h, and analyzed with GC-MS (Hewlett Packard 7890A and 5975C, Agilent Technologies) equipped with a DB5-MS column (J&W Scientific). The GC-MS program was as follows: the temperature of the column was initially held at 150°C for 2 min, raised to 280°C at 3°C·min⁻¹ and then to 300°C at 20°C·min⁻¹ and held at 300°C for 5 min. One µl of the sample was injected and flowed into the column at 1.2 ml·min⁻¹ at a 1:20 split ratio using helium as the carrier gas. The mass spectra were analyzed using Enhanced Data Analysis software (Agilent Technologies). Mass isotopomer distributions were obtained by integration and corrected for natural isotope abundances (Leighty and Antoniewicz, 2012).

Metabolic Flux Analysis

¹³C-MFA was performed using the Metran software (Yoo et al., 2008), which is based on the elementary metabolite units (EMU) framework (Antoniewicz et al., 2007a; Young et al., 2008). The *E. coli* network model used for flux calculations was described previously (Leighty and Antoniewicz, 2012) and is given in Supplementary Table I. In brief, the model included all major reactions of the central carbon metabolism, fatty acid production, amino acid biosynthesis, lumped biomass formation, and transhydrogenation reaction. The model accounts for the exchange of intracellular and extracellular CO₂ (Leighty and Antoniewicz, 2012) and includes G-value parameters to describe fractional labeling of amino acids (Antoniewicz et al., 2007b). One G-value parameter was included for each measured amino acid in each data set. Metabolic fluxes were estimated by minimizing the variance-weighted sum of squared residuals (SSR) between the experimentally measured and model predicted extracellular rates and mass isotopomer distributions using non-linear least-squares regression (Antoniewicz et al., 2006). For combined analysis of parallel labeling experiments, the data sets were fitted simultaneously to a single flux model (Leighty and Antoniewicz, 2012; Crown and Antoniewicz, 2013). Flux estimation was repeated at least 10 times starting with random initial values for all fluxes to find a global solution. At convergence, accurate 68% and 95% confidence intervals were computed for all estimated fluxes by evaluating the sensitivity of the minimized SSR to flux variations (Antoniewicz et al., 2006). Standard deviations of fluxes were determined as follows (Antoniewicz et al., 2006):

$$\text{Flux precision (SD)} = [(\text{flux}_{\text{upper bound 95 \%}}) - (\text{flux}_{\text{lower bound 95 \%}})]/4 \quad (1)$$

To determine the goodness-of-fit, ¹³C-MFA fitting results were subjected to a χ^2 -statistical test. In short, the minimized SSR value is a stochastic variable with a χ^2 -distribution, where the number of degrees of freedom is equal to the number of fitted measurements n minus the number of estimated independent parameters p . The acceptable range of SSR values is

between $\chi^2_{\alpha/2}(n-p)$ and $\chi^2_{1-\alpha/2}(n-p)$, where α is a certain chosen threshold value, for example 0.05 for 95% confidence interval (Antoniewicz et al., 2006).

qRT-PCR

RNAs were extracted from exponentially growing cells in baffled flasks in duplicate using RNeasy mini kit (Qiagen). Contaminating DNA was removed with RNase-free DNase I (Fermentas). The purified RNAs were quantified using a NanoDrop 200C spectrophotometer (Thermo Scientific). cDNAs were synthesized using a Revert Aid First Strand cDNA Synthesis Kit (Thermo Scientific) with random hexamer primers following the manufacturer's protocol supplied. The synthesized cDNAs were diluted 5-fold in nuclease-free water, and 2 μ l was amplified using the Power SYBR Green PCR Master Mix (Applied Biosystems) and primers specific to the genes of interest (Supplementary Table II) in a 20 μ l reaction system. The reaction for each gene in each sample was performed in triplicates. qRT-PCR assays were carried out on an ABI7500 fast machine with the thermal cycling conditions recommended by the manufacturer. For data analysis, expression levels of the house keeping gene *dnaK* were used as a control for normalization between samples. Fold changes of genes of interest were calculated as 2^{-CT} according to Schmittgen and Livak (2008).

Results

Growth Characteristics of the Fatty Acid Producing *E. coli*

To characterize the growth behavior of the fatty acid producing *E. coli* and investigate the effect of fatty acid overproduction, both the engineered (*E. coli* DH1 *fadE*/pA58C-TR) and the control (*E. coli* DH1 *fadE*/pACYCDuet-1) strains were grown in batch cultures on glucose under aerobic conditions. Fig. 2 shows the growth curves of the two strains and Table I summarizes the growth parameters. Both strains converted glucose to biomass, CO₂, acetic acid and fatty acids without any other major by-products. The engineered strain consumed 17.9 g·l⁻¹ glucose, with a slightly higher glucose consumption rate compared to the control strain, and produced 3.05 g·l⁻¹ of fatty acids at the end of the exponential phase in 23 h, corresponding to the yield of 0.17 g·g glucose⁻¹ (~ 48% of maximum theoretical yield). The control strain consumed 8.2 g·l⁻¹ of glucose and produced only 0.2 g·l⁻¹ of fatty acids (0.024 g·g glucose⁻¹). Free fatty acids produced by the engineered strain were composed of chain lengths ranging from C12 to C18, whereas the control strain mainly produced C16:0 free fatty acids (Fig. 3). Acetic acid yield was 10-fold lower in the engineered *E. coli* strain (0.011 g·g glucose⁻¹) compared to the control strain (0.11 g·g glucose⁻¹). The engineered *E. coli* had a lower specific growth rate (0.26 h⁻¹) and a lower biomass yield (0.21 g/g glucose) compared to the control strain (0.52 h⁻¹ and 0.43 g·g glucose⁻¹).

Metabolic Flux Distributions

Metabolic fluxes (Fig. 4; Supplementary Table IV) were determined based on the measured mass isotopomer distributions of proteinogenic amino acids (Supplementary Results; Supplementary Table III; Supplementary Fig. 2) and production rates of acetic acid and fatty acids (Table I). The measured biomass formation rates (Table I) were not employed as the

constraints of the ^{13}C -MFA model; instead, they were estimated by ^{13}C -MFA and then compared to the measured value to validate the metabolic flux results. As can be seen in Fig. 4, fluxes in the central metabolism of *E. coli* were redistributed in response to fatty acid overproduction. Firstly, the flux portioning at the acetyl-CoA node was strikingly different between the engineered and control strains. In the engineered strain, the flux into fatty acid production was boosted 7.8-fold, whereas the flux into acetic acid production decreased 10-fold, and the flux into the TCA cycle also decreased slightly. Secondly, the flux through the PP pathway increased 1.5-fold in the engineered strain. Thirdly, the flux through the Entner-Doudoroff (ED) pathway increased 2-fold. The co-regulation of PP and ED pathways is not a coincident; it has been reported that the alleles encoding *zwf* (G6P dehydrogenase encoding gene) in the PP pathway is in the same cluster as the *eda* gene (encoding 6-phosphogluconate dehydrogenase) in the ED pathway (del Castillo et al., 2007; Egan et al., 1992). The up-regulation of the ED pathway in the engineered *E. coli* enabled a direct supply of Pyr without carbon loss as CO_2 via the oxidative PP pathway ($6\text{PG} \rightarrow \text{Ru5P} + \text{CO}_2$). Fourthly, the flux through Ppc ($\text{PEP} \rightarrow \text{OAC}$), the major anaplerotic flux into the TCA cycle, decreased 1.7-fold in the engineered strain. The decrease of the Ppc flux is beneficial because it enabled more carbon flow from glucose to acetyl-CoA. The reduced Ppc flux could be related to the reduced demand for oxaloacetate based biomass synthesis (Table I). Fifthly, the estimated biomass biosynthesis flux was significantly lower in the engineered *E. coli*, nearly half of that in the control strain (Fig. 4), which correlated well with the decreased biomass yield (2-fold; Table I). Lastly, the transhydrogenation flux ($\text{NADH} \rightarrow \text{NADPH}$) was unregulated 1.7-fold.

Relative Gene Transcription Levels

To investigate the transcriptional response to the fatty acid overproductions, seven selected genes that are either located at the pathway branches or related to NADPH production were analyzed by qRT-PCR (Fig. 5). Compared to the control strain, the transcription levels of genes at acetyl-CoA node, *ackA* (encoding acetic acid kinase) and *gltA* (encoding citrate synthase), were lower, suggesting reduced metabolic activities in acetic acid production and TCA cycle. In contrast, *zwf*, encoding the first enzyme in the PP pathway, G6P dehydrogenase, showed higher expression levels, indicating the elevated PP pathway activity. In addition, the transcription levels of *icd* (encoding isocitrate dehydrogenase) and *maeB* (encoding malic enzyme) were higher in the engineered strain. Moreover, the transcription levels of *udhA* (encoding soluble transhydrogenase) and *pntA* (encoding membrane-bound transhydrogenase) were significantly higher in the engineered strain, increased 1.4- and 2.1-fold, respectively. Taken together, the transcription results suggested that there was an elevated activity in NADPH production pathways in the engineered *E. coli*, as evidenced by the up-regulation of *zwf*, *icd*, *maeB*, *pntA*, *udhA* genes that all encode the enzymes catalyzing relations with NADPH production. Compared to the flux results, the changes of gene transcription levels of *ackA*, *gltA*, *pntA*, *udhA* and *zwf* agreed with the changes of fluxes (Fig. 4). However, there was inconsistency between the gene transcription level and metabolic flux in the case of *maeB*, the transcription level of which increased about 4-fold but the corresponding metabolic flux did not change in the same manner, indicating the complex regulations through the interactions of genes, proteins and metabolites at multiple regulatory levels. The increase of *maeB* transcription was probably

induced by stress response mechanisms caused by the imbalance in NAD(H)/NADP(H) ratio in the engineered *E. coli* due to fatty acid production (Wang et al., 2011). On the other hand, the low malic enzyme flux could be attributed to the low substrate availability in the engineered *E. coli* due to the redirection of carbon fluxes to fatty acid production and/or post-translational protein modifications. The observation that gene expression is not always correlating with the corresponding metabolic flux has previously been reported (Hua et al., 2007), suggesting that gene transcription analysis alone could not sufficiently predict the metabolic responses of the entire organism to genetic perturbations (Moxley et al., 2009).

Discussion

Phenotype of the Fatty Acid Producing *E. coli*

The phenotype of the engineered *E. coli* is characterized as follows: 1) a typical growth pattern of *E. coli* was observed with an obvious exponential phase after IPTG induction; 2) the exponential cell growth rate ($\mu = 0.26 \text{ h}^{-1}$) and biomass yield ($Y_{x/G} = 0.20 \text{ g} \cdot \text{g}^{-1}$) were significantly lower compared to the control strain ($\mu = 0.52 \text{ h}^{-1}$, $Y_{x/G} = 0.43 \text{ g} \cdot \text{g}^{-1}$); 3) during the exponential growth phase, fatty acid production was dominant with little acetic acid secreted; 4) during the stationary phase, cell density remained constant and fatty acid production was insignificant, whereas, acetic acid production showed a remarkable increase.

The engineered *E. coli* strain produced $3.05 \text{ g} \cdot \text{l}^{-1}$ and $0.17 \text{ g} \cdot \text{g} \text{ glucose}^{-1}$ of fatty acids corresponding to 48% of the maximum theoretical yield. This result was comparable to the recent study by Zhang et al. (2012b) that used a similar genetic strategy, considering our data were collected in 23 hours instead of after 3 days. The observed chain lengths of the fatty acids produced from this study were dominated by saturated fatty acids C14:0 and C16:0 (Fig. 3). Compared to the strain constructed by Zhang et al. (2012b), which produced a higher amount of C16:1 fatty acid, the lower unsaturated fatty acid contents observed in our study is consistent with the lower FadR copy number (in a p15A plasmid, Supplementary Fig. 1) used in our strain. Like the previous study by Steen et al. (2010), we detected a small amount of 17:0 cyclopropane fatty acid, which was presumably the result of methylation of C16:1 fatty acid induced by free fatty acid accumulation (Cao et al., 2010). In addition, we observed that cell cultures inoculated from different colonies exhibited varied fatty acid productivity (data not shown), which confirmed the self-mutagenesis when *E. coli* was accumulating free fatty acids (Lennen et al., 2011).

Central Carbon Metabolic Fluxes

We performed ^{13}C tracer experiments and ^{13}C -MFA for the two strains at each time point during the exponential growth phase of each culture (Supplementary Results; Supplementary Table IV). We obtained statistically acceptable flux estimations in all cases (Supplementary Table V), and the flux results were proven to be reliable as demonstrated by the agreement of the ratios of the estimated biomass fluxes ($180/343 \approx 0.5$) and measured biomass yields ($143/302 \approx 0.5$) between the engineered and control strains (Supplementary Table VI). We observed that: 1) the mass isotopomer distributions of proteinogenic amino acids and metabolic fluxes were constant among the three time points during the exponential phase, indicating that the metabolism maintained a pseudo-steady state condition; 2) the fluxes

were reproducible between the two biological replicates, indicating constant cellular metabolism of the engineered *E. coli* and constant cultivation conditions maintained during the experiments; and 3) there were notable differences in the fluxes between the engineered and control strains, suggesting altered metabolism of the engineered *E. coli*. The central metabolism in the engineered strain reduced acetate secretion and biomass biosynthesis so that more acetyl-CoA was directed to fatty acid synthesis (One C16:0 fatty acid requires 8 acetyl-CoA). Moreover, the engineered strain reduced Ppc flux ($\text{PEP} \rightarrow \text{OAC}$) so that the PEP pool was retained for acetyl-CoA production. The reduced Ppc flux in the engineered *E. coli* could be related to slower biomass synthesis since the role of Ppc in *E. coli* is to replenish oxaloacetate for biomass synthesis (Sauer et al., 1999). In addition, both the PP and ED pathway fluxes were up-regulated. The up-regulation of ED pathway flux was more efficient for fatty acid production since it not only enhanced Pyr flux by providing a short path and avoiding carbon loss as CO_2 via the PP pathway ($6\text{PG} \rightarrow \text{Ru5P} + \text{CO}_2$), but also produced one NADPH through the shared G6P dehydrogenase catalyzed reaction. Overall, the carbon fluxes of the central metabolism in the engineered *E. coli* were redirected towards acetyl-CoA for fatty acid synthesis.

Cofactor NADPH Metabolism

The cofactor balancing can be estimated based on the metabolic fluxes (Kind et al., 2013; Sauer et al., 2004). The major pathways supplying NADPH are G6P and 6PG dehydrogenases in the PP pathway, and NADP^+ -dependent isocitrate dehydrogenase in the TCA cycle. Additionally, NAD(P) transhydrogenase, encoded by *pntAB* and *udhA* genes, can catalyze the reversible conversion between NADH and NADPH to balance the cofactors (Hua et al., 2003; Sauer et al., 2004). The relative contributions of these pathways to NADPH production in the engineered and control strains are shown in Table II. As can be seen, the PP pathway contributed to the supply of NADPH in a large fraction in both *E. coli* strains, which was further up-regulated 1.5-fold in the engineered strain to increase NADPH production. This result is consistent with previous studies on other *E. coli* strains (Emmerling et al., 2002; Hua et al., 2003; Sauer et al., 2004) as well as *C. glutamicum* for lysine production that requires NADPH as the cofactor (Kind et al., 2013). The fluxes from the PP pathway and TCA cycle in the central metabolism added up to a total NADPH supply of $100 \text{ mol-mol glucose}^{-1}\cdot\text{h}^{-1}$, accounting for 50% of the NADPH consumption in the engineered strain (Table II). Thus, there was a 50% gap to be filled to satisfy NADPH demand without perturbing the carbon balances. The transhydrogenation reaction served an excellent role for this purpose. Our flux analysis indicated a significant transhydrogenation flux in the engineered strain (Fig. 4), accounting for 76% of NADPH consumption for fatty acids and biomass synthesis. Compared to the control strain, the transhydrogenation flux increased 1.7-fold in the engineered strain (Fig. 4), which was consistent with the up-regulated transcription levels of *pntA* and *udhA* genes (Fig. 5). Thus, transhydrogenation reaction appeared to play an important role in supplying NADPH for fatty acid production in the engineered *E. coli*. As revealed by the ^{13}C -MFA and cofactor balancing analysis, *E. coli* has the ability of replenishing NADPH that is consumed in biomass synthesis and fatty acid production through enhancing the transhydrogenation reaction ($\text{NADP}^+ + \text{NADH} \rightarrow \text{NAD}^+ + \text{NADPH}$) and PP pathway fluxes. This finding is consistent with the previous research that

engineering NADPH supply in *E. coli* is unlikely to enhance fatty acid productivity, obtained in a cell free system (Liu et al., 2010).

Energy ATP Metabolism

Based on the fluxes, ATP formation and consumption for cell growth and non-growth associated cellular maintenance of the two strains were estimated (Table II). The ATP formation fluxes through the glycolysis, TCA cycle and acetic acid secretion pathways added up to 157 and 153 mol-mol glucose⁻¹·h⁻¹ in the engineered and control strains, respectively. Another route of ATP production was via oxidation phosphorylation through the respiratory chain. Assuming the maximum P/O ratio (the moles of ATP formed per oxygen atom: NADH → 3ATP, FADH₂ → 2ATP, Mitsumori et al., 1988), the ATP formation fluxes via oxidation phosphorylation were 664 mol-mol glucose⁻¹·h⁻¹ in the engineered and 782 mol-mol glucose⁻¹·h⁻¹ in the control strain. In total, there were 821 and 935 mol-mol glucose⁻¹·h⁻¹ of ATP formed in the engineered and the control strains, respectively, which were more than enough for cell growth and fatty acid production demands (266 in the engineered and 350 in control were demanded, Table II). The large portions of the ATP produced, 555 mol-mol glucose⁻¹·h⁻¹ in the engineered *E. coli* and 585 mol-mol glucose⁻¹·h⁻¹ in the control strain, were potentially consumed for non-growth associated cellular maintenance. When expressed on the basis of dry cell weight (Table I), the cellular maintenance ATP was 154 mmol-g DCW⁻¹·h⁻¹ in the engineered *E. coli* and 76 mmol-g DCW⁻¹·h⁻¹ in the control strain. Clearly, the engineered strain required much higher cellular maintenance energy compared to the control strain (Youngquist et al., 2012).

Cellular maintenance covers every cellular reaction involving the consumption of ATP that does not contribute to the net synthesis of biomass and product. Such reactions include the building-up and maintenance of ionic gradients across the membrane and regeneration of degraded macromolecules (Stephanopoulos et al., 1998). Osmotic stress resulting from the culture medium and product accumulation, and disrupted bacterial growth are the major stress factors causing increased cellular maintenance energy (Varela et al., 2004; Varela et al., 2003). Previous study found that the overproduction of fatty acids in *E. coli* caused the change in membrane properties due to fatty acid intercalation in the inner or outer membranes, fatty acid accumulation in the periplasm, and change of the composition of membrane lipids (Lennet et al., 2011). These changes incurred secondary effects on the cell including induced membrane stress, compromised membrane integrity, and reduced cell viability (Lennet et al., 2011). In addition, intracellular fatty acid accumulation could counteract ion diffusion into the cytoplasm. These factors could be likely responsible for the higher cellular maintenance energy observed in the engineered *E. coli* to form and sustain ionic gradients across the cell membrane.

Analysis of energy metabolism (Table II) also revealed that oxidative phosphorylation of NADH played an important role in supplying ATP to support cell growth and maintenance of the engineered *E. coli*. Therefore, it is important to maintain high respiration efficiency (i.e., the P/O ratio) during the cultivation in the future research. To this end, the culture condition such as the glucose concentration should be optimized and the dissolved oxygen

concentration in the culture should be precisely monitored and controlled during the cultivation (Noguchi et al., 2004).

Significance of This Study and Future Metabolic Engineering Strategies

The “pull-push-block” strategies, targeted on engineering genes in fatty acid metabolic pathways, have achieved *E. coli* fatty acid production with less than 70% of the maximum theoretical yield. Further enhancement has proven unpredictable due to the challenges in balancing the precursor acetyl-CoA, cofactor NADPH and energy ATP in the engineered *E. coli* that are mainly derived in the central metabolism (Lennen and Pfleger, 2012). To decipher the metabolic bottlenecks, previously “omics” techniques were employed to compare the profiles of genes and proteins between the fatty acid producing strain and the control strain (Lennen et al., 2011; Zhang et al., 2012b). Unfortunately, only a limited number of genes and enzymes involved in the central metabolism were detected and no information on the reaction rates was provided in these investigations. The overall *E. coli* central metabolism and how it is regulated for fatty acid production remained unclear. In this study, we used ^{13}C -MFA to quantify the *E. coli* metabolism and its regulations in response to fatty acid overproduction. ^{13}C -MFA revealed the flexibility of the central metabolism at the nodes of PP pathway, PEP carboxylation, acetic acid secretion and TCA cycle to accommodate fatty acid overproduction. ^{13}C -MFA also revealed that the reversible transhydrogenation reaction in *E. coli* could be significantly regulated to balance the cofactors (NADH and NADPH) to meet with the NADPH requirement for fatty acid production. In contrast, ^{13}C -MFA found that the ED pathway flux was inherently low despite of 2-fold upregulation in the fatty acid producing *E. coli* (Fig. 4), which appeared to be a rigid node. The upregulation of the ED pathway in the engineered strain is beneficial to fatty acid production since it enhance the supply of pyruvate, the substrate of acetyl-CoA, by avoiding carbon loss as CO_2 through the PP pathway while providing one NADPH though G6P dehydrogenase. It is generally believed that rigid nodes are promising targets for metabolic engineering while flexible nodes are poor targets (Stephanopoulos and Vallino, 1991). Therefore, the ED pathway could be a promising target that could be overexpressed in combination with the “pull-push-block” strategy to improve fatty acid production. As demonstrated in this study, the acetate formation pathway was a flexible node and knocking out acetate formation pathway did not improve fatty acid production (Li et al., 2012).

^{13}C -MFA based energy balance analysis revealed that a large fraction of ATP supply relied on phosphorylation of NADH via respiration. However, engineered microbial strains often have lower P/O ratio, which limits the high yield of final products (Sauer and Bailey, 1999). To enhance the respiration efficiency, one strategy would be overexpression of NADH dehydrogenase. The aerobic respiratory chain of *E. coli* functions with either of the two different membrane-bound NADH dehydrogenases, NDH-1 (encoded by *nuoA-N*) and NDH-2 (encoded by the *ndh*), coupled with the *bd*-type or *bo*-type ubiquinol oxidases (Calhoun et al., 1993). Therefore overexpressing NDH-1 (or NDH-2) and *bo*-type oxidase (*bd*-type is less efficient) could be an effective approach to increase the P/O ratio. Meanwhile, the dissolved O_2 concentration in the culture should be controlled to maintain respiration efficiency since these enzymes are regulated in O_2 -dependent manner (Calhoun et al., 1993; Noguchi et al., 2004). This study also revealed that the fatty acid producing *E.*

coli required high maintenance energy, which is likely due to fatty acid accumulation that interfered with the ion transport and cell membrane function. Overexpressing fatty acid exporter genes and repressing *fabR* (the gene controlling the unsaturated fatty acid biosynthesis according to the ratio of unsaturated to saturated-ACPs in the membrane lipid and restores the lipid composition of the membrane) could reduce the undesired effect of fatty acid accumulation on cell membrane function (Lennen et al. 2011).

Conclusions

This work is an evident example that ^{13}C -MFA is a powerful strategy for quantitative investigation of engineered microbes to provide metabolic insights. ^{13}C -MFA illustrates the high flexibility of the metabolic network of *E. coli* to compensate for external perturbation, and meanwhile identifies the rigid nodes for future metabolic engineering. Moreover, ^{13}C -MFA based cofactor and energy balance analyses allow the evaluation of the energy status of the cells, which can lead to additional findings. This work provides important information about the metabolic bottlenecks of *E. coli* overproducing fatty acids and strategies which could be exploited in combination of the “pull-push-block” approach for future strain development and bioprocess optimization for the enhancement of fatty acid production.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Fatty acid metabolism of *E. coli* and the “pull-push-block” strategy for fatty acid production. Overexpression of *tesA* and *fadR* pulls and pushes the carbon fluxes to fatty acid production, while knockout of *fadE* blocks fatty acid degradation. Red-boxed genes are positively regulated by FadR. Green-boxed genes are negatively regulated by the *fadR* gene product, FadR. Bolded arrows represent reactions enhanced by overexpressing the indicated genes. × represents inactivation of the corresponding pathway by knocking out the indicated gene. T represents repression or activation exerted by FadR. Full names of genes: *accABCD*, acetyl-CoA carboxylase; *ackA*, acetate kinase A; *fabA*, beta-hydroxydecanoyl thioester dehydratase; *fabB*, 3-oxoacyl-ACP synthase I; *fabD*, malonyl-CoA ACP transacylase; *fabF*, 3-oxoacyl-ACP synthase II; *fabG*, 3-oxoacyl-ACP reductase; *fabH*, 3-oxoacyl-ACP synthase III; *fabI*, enoyl-ACP reductase; *fabZ*, (3R)-hydroxymyristoyl acyl carrier protein dehydratase; *fadA*, 3-ketoacyl-CoA thiolase; *fadB*, 3-hydroxyacyl-CoA dehydrogenase; *fadD*, acyl-CoA synthetase; *fadE*, acyl coenzyme A dehydrogenase; *plsB*, glycerol-3-phosphate O-acyltransferase; *pta*, phosphate acetyltransferase; *tesA*, acyl-ACP thioesterase.

Figure 2.

Growth curves of the engineered fatty acid producing strain *E. coli* DH1 *fadE/pA58C-TR* (A) and the control strain DH1 *fadE/pACYCDuet-1* (B) in batch cultures grown on glucose under aerobic conditions. Arrows indicate the time points of IPTG addition or sampling for isotopic labeling patterns of proteinogenic amino acids, mRNA and external metabolites analyses. Square, Glucose; Diamond, Biomass; Triangle, Acetic acid; Circle, Fatty acid. Results are from parallel cultivations.

Figure 3.

Free fatty acid profiles produced by the engineered fatty acid producing *E. coli* strain (A) and the control strain (B). Symbols a, b and c represent the sampling time points for fatty acid analysis as indicated in Fig. 2.

Figure 4.

Metabolic flux distributions in the central metabolic pathways of the engineered fatty acid producing *E. coli* strain and the control strain during exponential growth on [1,2-¹³C]glucose. Fluxes shown are normalized to glucose uptake rate of 100 for each strain (estimated flux $\pm$ SD). Abbreviations: G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose 1,6-bisphosphate; DHAP, Dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; 3PG, 3-phosphoglycerate; PEP, phosphoenolpyruvate; Pyr, pyruvate; AcCoA, acetyl coenzyme A; Cit, citrate; ICit, isocitrate; AKG, α -oxoglutarate; Suc, succinate; Fum, fumarate; Mal, malate; OAC, oxaloacetate; Glyox, glyoxylate; 6PG, 6-phosphogluconate; Ru5P, ribulose-5-phosphate; X5P, xylulose-5-phosphate; R5P, ribose-5-phosphate; E4P, erythrose-4-phosphate; S7P, seduheptulose-7-phosphate; TK-C2, the first two carbon unit of F6P or S7P; TA-C3, the first three carbon unit of F6P or S7P; KDPG, 2-keto-3-deoxy-6-phosphogluconate. The reactions are given in Supplementary Table I.

Figure 5. Fold changes of transcript levels of the selected genes in the engineered fatty acid producing *E. coli* strain compared to the control strain. *ackA*, acetate kinase A; *gltA*, citrate synthase; *icd*, isocitrate dehydrogenase; *maeB*, malic enzyme; *pntA*, membrane-bound transhydrogenase; *udhA*, soluble transhydrogenase; *zwf*, G6P dehydrogenase.

Exponential growth parameters of the engineered and control *E. coli* strains grown on glucose in minimal medium. $Y_{X/G}$, biomass yield on glucose; $Y_{F/G}$, fatty acid yield on glucose; $Y_{A/G}$, acetic acid yield on glucose. These values were determined from the mean values of parallel cultivations corresponding to Fig. 2.

Table 1

Strain	Specific growth rate (h^{-1})	Specific rate ($mmol \cdot g \text{ DCW}^{-1} \cdot h^{-1}$) of			Yield ($g \cdot g^{-1}$)		
		Glucose uptake	Fatty acid formation	Acetic acid formation	$Y_{X/G}$	$Y_{F/G}$	$Y_{A/G}$
Engineered	0.26	7.2	0.89	0.24	0.20	0.17	0.011
Control	0.52	6.6	0.10	2.2	0.43	0.022	0.11

Table II

Estimated production and consumption of NADPH, NADH, ATP and FADH₂ for the *E. coli* control and engineered strains. All the flux values are normalized to the glucose uptake rate of 100 mol.h⁻¹ for each strain.

Pathways	NADPH		NADH		ATP		FADH ₂	
	Control	Engineered	Control	Engineered	Control	Engineered	Control	Engineered
Glycolysis	0	0	167	171	107	141	0	0
PP pathway	54	80	0	0	0	0	0	0
TCA cycle	27	20	141	177	13	13	19	17
Amino acid synthesis	-103	-54	17	9	-52	-28	0	0
One-carbon metabolism	0.8	0.4	0	0	0	0	0	0
Fatty acid formation	-23	-177	0	0	-11	-88	0	0
Acetic acid formation	0	0	0	0	33	3	0	0
Biomass formation	-46	-24	13	7	-287	-150	0	0
Transhydrogenation	90	153	-90	-153	0	0	0	0
Oxidative phosphorylation [*]	0	0	-248	-210	782	664	-19	-17

^{*} Note: The excessive NADH and FADH₂ were assumed to be converted to ATP via oxidation phosphorylation at the maximum P/O ratio (NADH → 3ATP, FADH₂ → 2ATP) (Mitsumori et al., 1988).