Metabolic Engineering 14 (2012) 687–704

Contents lists available at SciVerse ScienceDirect

Metabolic Engineering

journal homepage: www.elsevier.com/locate/ymben

An integrated computational and experimental study for overproducing fatty acids in *Escherichia coli*

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ARTICLE INFO

Article history:
Received 14 May 2012
Received in revised form
11 July 2012
Accepted 8 August 2012
Available online 2 October 2012

Keywords:
Fatty acids overproduction
Computational strain design
Metabolic flux analysis

ABSTRACT

Increasing demands for petroleum have stimulated sustainable ways to produce chemicals and biofuels. Specifically, fatty acids of varying chain lengths (C_6–C_{16}) naturally synthesized in many organisms are promising starting points for the catalytic production of industrial chemicals and diesel-like biofuels. However, bio-production of fatty acids from plants and other microbial production hosts relies heavily on manipulating tightly regulated fatty acid biosynthetic pathways. In addition, precursors for fatty acids are used along other central metabolic pathways for the production of amino acids and biomass, which further complicates the engineering of microbial hosts for higher yields. Here, we demonstrate an iterative metabolic engineering effort that integrates computationally driven predictions and metabolic flux analysis techniques to meet this challenge. The OptForce procedure was used for suggesting and prioritizing genetic manipulations that overproduce fatty acids of different chain lengths from C_6 to C_{16} starting with wild-type *E. coli*. We identified some common but mostly chain-specific genetic interventions alluding to the possibility of fine-tuning overproduction for specific fatty acid chain lengths. In accordance with the OptForce prioritization of interventions, *fabZ* and acyl-ACP thioesterase were upregulated and *fadD* was deleted to arrive at a strain that produces 1.70 g/L and 0.14 g fatty acid/g glucose (~39% maximum theoretical yield) of C_{14-16} fatty acids in minimal M9 medium. These results highlight the benefit of using computational strain design and flux analysis tools in the design of recombinant strains of *E. coli* to produce free fatty acids.

1. Background and introduction

The economical production of industrial chemicals (Nikolau et al., 2008) and transportation fuels (Stephanopoulos, 2007) from renewable resources is advancing but remains a grand challenge. In particular, microbial synthesis of free fatty acids for the production of biorenewable chemicals and fuels has garnered extensive interest recently ((Nikolau et al., 2008); (Steen et al., 2010); (Handke et al., 2011); (Liu et al., 2010a)). First generation biofuels, such as bio-ethanol (Fortman et al., 2008) produced from corn has relatively low energy density and water miscibility. New efforts are focused on longer chain alcohols such as 1-butanol (Gulevich et al., 2011; Lan and Liao, 2011; Shen and Liao, 2008), isobutanol (Atsumi et al., 2010) and 1,3-butanediol (Gonzalez et al., 2010) as gasoline bio-alternatives and fatty acids as promising intermediates for diesel bio-alternatives (Lu et al., 2008). Fatty acids produced during fermentation can be converted to alkanes by catalytic esterification or decarboxylation (Fjerbaek et al., 2009; Vasudevan and Briggs, 2008). Conversely, the host organism could be bioengineered to convert fatty acids towards fatty acid ethyl esters (FAEE) (Steen et al., 2010) which have high energy density and low water solubility (Atsumi et al., 2010). Medium chain fatty acids (C_6–C_{14}) find attractive industrial applications as sources for detergents, lubricants, cosmetics, and pharmaceuticals. Free fatty acids can be directly hydrogenated to form fatty alcohols (Voeste and Buchold, 1984). More recently, it has been shown that fatty acids could be catalytically deoxygenated via Pd or Rh catalysts (George Kraus, unpublished results) to produce α-olefins, which serve as building blocks of important polymerization products. In addition, existence of plant thioesterases that can specifically hydrolyze acyl-ACP substrates of a particular chain length (Jing et al., 2011) creates the opportunity to produce novel fatty acids.

Most bacteria are naturally equipped to produce fatty acids as an important constituent of their cell envelopes (Magnuson et al., 1993), however, transcriptional and post-transcriptional control in *Escherichia coli* tightly regulates the metabolism of fatty acid

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http://dx.doi.org/10.1016/j.ymben.2012.08.008

biosynthesis. The two most important metabolic steps of fatty acid synthesis are the conversion of acetyl-CoA into malonyl-CoA by an ATP-dependent acetyl-CoA carboxylase (ACCOAC) and the conversion of malonyl-CoA into fatty acid product bound to an acyl carrier protein (ACP) by a multi-subunit synthase (see Fig. 1). Fatty acids produced from acetyl-CoA and malonyl-ACP are primarily used for phospholipid biosynthesis and synthesis of lipid A and coenzyme A (CoA) intermediates in *E. coli* (Dellomonaco et al., 2011; Gulevich et al., 2011; Poirier et al., 2006). Overproduction of fatty acids in *E. coli* is however a difficult challenge because of tight transcriptional and post-transcriptional regulation of fatty acid biosynthesis (Fujita et al., 2007; Magnuson et al., 1993; White et al., 2005) including strong product inhibition (Gonzalez et al., 2010). For example, *fabH* and *fabI*, which encode the acyl-ACP synthesis and reduction of enoyl-ACP reaction steps (i.e., ketoacyl–ACP synthase (KAS15) and enoyl–ACP reductase), respectively, are also inhibited by long chain fatty acyl-ACPs (Heath and Rock, 1996a, 1996b).

Inspite of these difficulties, recent efforts have led to improvements in the synthesis of free fatty acids in *E. coli*; however, most of the reported engineering strategies rely on manipulating terminal pathways near the target fatty acid. This trend may be due to the complexity of metabolism and the difficulty of predicting the effect of manipulations at a systemic level. Genetic interventions and strategies reported to date include blocking fatty acid degradation through the β-oxidation pathway by knocking out *fadD* or *fadE* genes (Lu et al., 2008), heterologous expression of thioesterase genes from *U. californica* (Lu et al., 2008) or *C. camphorum* (Lu et al., 2008) to target fatty acids of specific chain lengths (Dehesh et al., 1996; Liu et al., 2010b; Nawabi et al., 2011), and augmenting the availability of precursors by overexpressing acetyl-CoA carboxylase (Lennen et al., 2010). Most of these approaches have resulted in *E. coli* strains that show selectivity towards the production of C_{14-16} fatty acids. Alternatively, Dellomonaco et al. (2011) recently reported on an engineered reversal of the β-oxidation pathway in *E. coli* leading to a significant increase in the production yield of 1-butanol as well as a number of long chain fatty acids. We note that even though the reported yields for middle chain-length fatty acids using the chain elongation (type II fatty acid synthesis) pathway and reversed β-oxidation pathway are quite similar, there are a number of differences in biosynthesis of fatty acids using these two pathways. In particular, the type II fatty acid synthesis pathway involves addition of two carbons from malonyl-ACP to the acyl-ACP skeleton in each cycle, along with the release of one molecule of CO_2. In contrast, in the reversed β-oxidation pathway

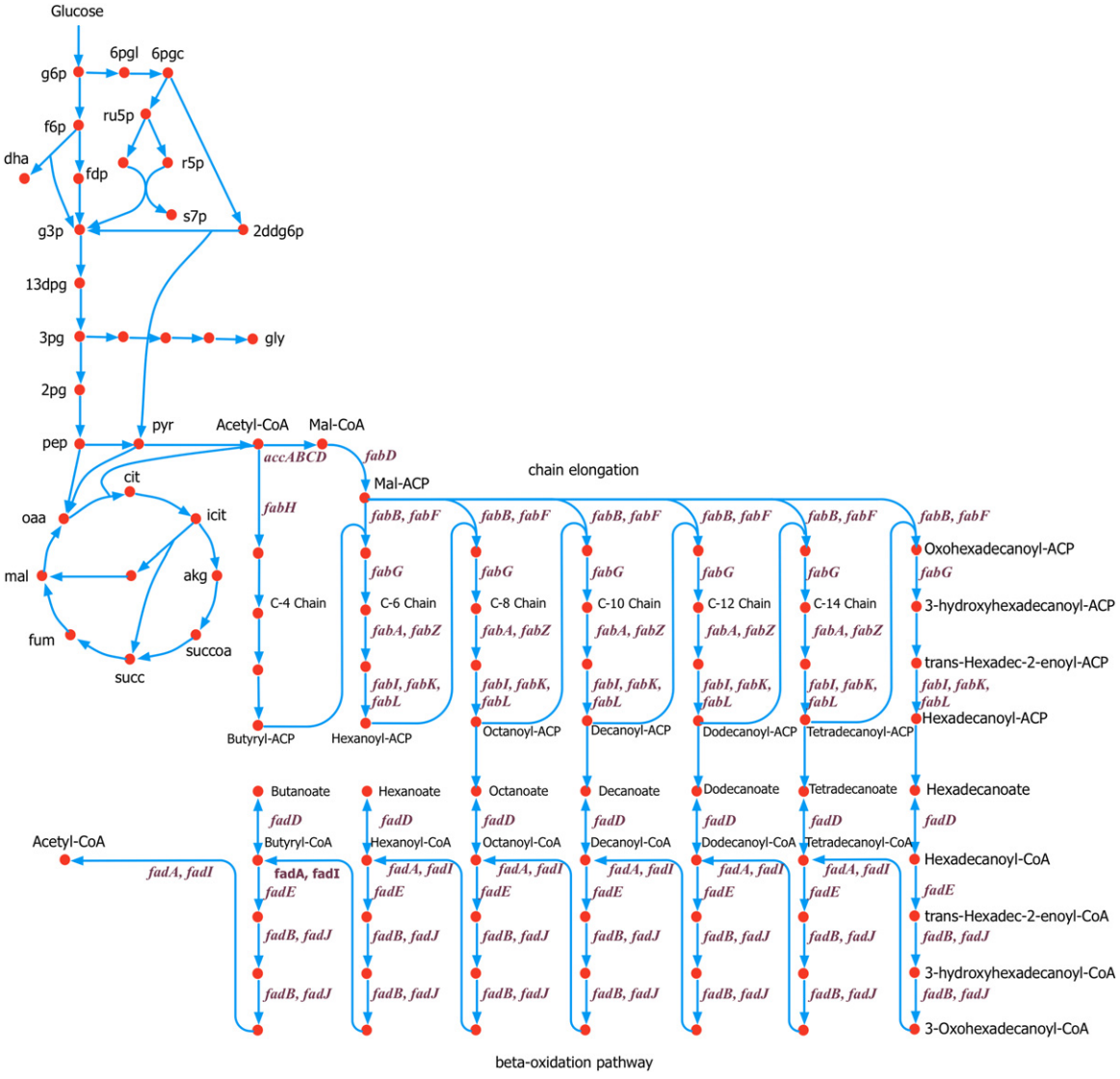

Fig. 1. Organization of fatty acid biosynthetic pathways in *E. coli* metabolism according to *i*AF1260 model.

one mole of acetyl-CoA is recruited in each cycle to increase the fatty acid chain length by two carbon units. In addition, the reduction steps in chain elongation pathway require NADPH as the cofactor while those for the reversed β-oxidation pathway utilize NADH and $FADH_2$. Finally, one ATP is needed to form each mole of malonyl-CoA in the type II synthesis pathway whereas no ATP is involved in the reversed β-oxidation pathway. Despite recent improvements, the production yield of fatty acids remains far below that of alcohols (Magnuson et al., 1993). For example, the maximum yield of fatty acid production by the type II synthesis pathway was recently reported to be 14% of the maximum theoretical yield (Steen et al., 2010), while that for alcohols is close to 70% (Magnuson et al., 1993).

In the past decade, a number of optimization procedures have been proposed to identify targets for gene knockouts (e.g. OptKnock (Burgard et al., 2003), RobustKnock (Tepper and Shlomi, 2010), OptORF (Kim and Reed, 2010), OptGene (Patil et al., 2005)), up-/down-regulations (e.g. OptReg (Pharkya and Maranas, 2006)) and knock-ins (i.e. OptStrain (Pharkya et al., 2004)) that lead to overproduction of specific biochemicals in microorganisms. Inspite of their success stories, none of these procedures proactively make use of flux data to drive the strain design process. Instead, they rely on hypothesized biological fitness functions such as maximization of biomass and minimization of metabolic adjustments (Segre et al., 2002). Metabolic flux analysis (MFA) has been increasingly been used to quantify internal metabolic fluxes (Wiechert et al., 2001) in strain engineering projects. Metabolic fluxes provide a unique description of cellular physiology and a starting point for pinpointing genetic manipulations (Koffas and Stephanopoulos, 2005; Peebles et al., 2010; Stephanopoulos, 1999). The OptForce procedure (Ranganathan et al., 2010) was designed to make use of flux measurements available for the wild-type strain. OptForce first estimates the maximum range of flux variability for all the reactions in the metabolic network of the wild-type strain and overproducing network. By overlaying the two sets, a set of reactions is revealed whose flux must depart away from the wild-type range if the imposed overproduction target is to be met (i.e., MUST sets). This set of required changes in the network can be described with a single logic statement containing AND, OR operators linking reaction up/down manipulations. OptForce subsequently chooses from this subset of reactions a list of targets that must be actively engineered to guarantee a target yield for the desired product. The genetic interventions identified by OptForce are prioritized based on their impact on product yield improvement and alternate intervention scenarios can be explored. The OptForce paradigm was recently demonstrated by constructing a strain of *E. coli* that produces increased levels of intracellular malonyl-CoA (Xu et al., 2011a), an important precursor for fatty acids.

In this study, we employed the OptForce procedure to identify the most promising engineering interventions that lead to the overproduction of fatty acids C_6 through C_{16} in *E. coli*. We next report on the improved production yield of C_{14-16} fatty acids following the implementation of some prioritized OptForce interventions (i.e. up-regulation of *fabZ* and acyl-ACP thioesterase and *fadD* knockout). The computational results and experimental measurements presented in this paper demonstrate that *E. coli* metabolism can be reprogrammed for specific fatty acid chain lengths using an integrated computations and experimentation paradigm.

2. Materials and methods

2.1. Strains and plasmids

All strains and plasmids used in this work are listed in Table 1 and were gifted by Ka-Yiu San's lab at Rice University.

Table 1
List of strains and plasmids used.

	Relative genotype	Source or reference
Strain name		
MG1655	F$^-$ lamda$^-$ *ilvG*$^-$ *rfb*$^-$ *rph*$^-$	ATCC 47076
ML103	MG1655 (Δ*fadD*)	(Li et al., 2012)
MLK163	MG1655 (Δ*fadD*, Δ*sucC*)	(San et al., 2011)
Plasmid name		
pTrc99a	pTrc99a, cloning vector	Amersham Pharmacia
PXZ18	pTrc99a carrying an acyl thioesterase *R. communis*	(Zhang et al., 2011)
PXZ18z	pTrc99a carrying an acyl thioesterase *R. communis* and overexpressed *fabZ*	(San et al., 2011)

2.2. Metabolic flux analysis experiments

2.2.1. Strains and culture conditions

An *E. coli* MG1655 strain from glycerol frozen stock was streaked on Luria Broth (LB) plate and incubated overnight at 37 °C. A single colony from the plate was grown in 25 mL MOPS minimal medium (8.37 g/L MOPS powder (Sigma-Alrich, Saint Louis, MO), 0.72 g Tricine (Sigma-Alrich, Saint Louis, MO), 2.92 g/L NaCl, 0.51 g/L NH_4Cl, 1.6 g/L KOH, 0.215 mg/L Na_2SeO_3, 0.303 mg/L Na_2MoO_4, 0.17 mg/L $ZnCl_2$, 50.3 mg/L $MgCl_2$, 48.1 mg/L K_2SO_4, 0.348 g/L K_2HPO_4, and micronutrients containing 2.5 mg/L $FeCl_2 \cdot 4H_2O$, 92 μg/L $CaCl_2 \cdot 2H_2O$, 31 μg/L H_3BO_3, 20 μg/L $MnCl_2 \cdot 4H_2O$, 9 μg/L $CoCl_2 \cdot 6H_2O$, 2 μg/L $CuCl_2 \cdot 2H_2O$, and 48.4 μM HCl) supplemented with 1% glucose in an orbital shaker at 250 rpm until exponential phase. The culture then was centrifuged at 4000 rpm for 5 min at 4 °C. The supernatant were discarded, and the pellet was re-suspended in fresh MOPS medium. The appropriate quantity of the washed cell suspension was used to inoculate 400 mL of MOPS medium in the 500 mL bioreactor (INFORS HT, Switzerland) to a starting OD_{550} of 0.03. For better identification of fluxes, a mixture of uniformly labeled [U-^{13}C], first carbon labeled [1-^{13}C] and natural glucose was used for ^{13}C flux analysis (Fischer et al., 2004). Specifically, 10% U-^{13}C glucose, 25% 1-^{13}C glucose, and 65% of naturally labeled glucose was used as the tracer to final medium concentration of 1% glucose. About 500 μL of antifoam (Antifoam B Silicone Emulsion, J.T. Baker) aquatic solution (volume ratio antifoam: water = 1: 1) was added into the media to prevent foaming. The aerobic fermentation was conducted at 37 °C, with a gas flow rate at 0.6 ml/min and agitation speed of 600 rpm. The pH was controlled at 7.0 ± 0.05 by adding 1 M potassium hydroxide. The dissolved oxygen level was maintained above 50% of saturated levels to ensure aerobic conditions. The cells were harvested at mid-exponential phase after at least five generations to ensure metabolic and isotopic steady state.

Due to reports of strains carrying the plant acyl-ACP thioesterase being unstable (Zhang et al., 2011), an additional metabolic flux experiment was conducted for *E. coli* but at a reduced temperature and bioreactor agitation. Batch fermentations were performed for ML103 strain (MG1655 Δ*fadD*) using minimal M9 (0.8 g/L NH_4Cl, 0.5 g/L NaCl, 7.52 g/L Na_2HPO_4, 3.0 g/L KH_2PO_4, 0.24 g/L $MgSO_4$, 11.1 mg/L $CaCl_2$, 1 mg/L thiamine HCl, and trace elements containing 166.7 μg/L $FeCl_3 \cdot 6H_2O$, 1.8 μg/L $ZnSO_4 \cdot 7H_2O$, 1.2 μg/L $CuCl_2 \cdot 2H_2O$, 1.2 μg/L $MnSO_4 \cdot 2H_2O$, 1.8 μg/L $CoCl_2 \cdot 6H_2O$, and 0.223 mg/L $Na_2EDTA \cdot 2H_20$) medium supplemented with 1% glucose (20% U-^{13}C glucose and 80% 1-^{13}C glucose), 100 mg/L ampicillin, and antifoam. The fermentor was controlled at pH 7.0, 30 °C and 300 rpm agitation. As in the previous experiment, dissolved oxygen level was maintained above 50% of saturated levels and cells were harvested at mid-exponential phase after at least five generations to ensure isotopic steady state.

2.2.2. Analytical techniques

Cell biomass dry weight was determined by measuring optical density OD_{550} using a spectrophotometer (Genesys 20, Madison, WI). Cell dry weight was estimated by the correlation: 1 OD_{550}= 0.36 g cell dry weight/L (Choudhary et al., 2011). Biomass composition was determined based on literature data (Ingraham, 1983). Media samples were taken during the exponential growth and filtered through 0.22 μm pore sized nylon filters (P.J. Cobert Associates, Saint Louis, MO) and kept at −20 °C for extracellular metabolite analysis. Glucose and acetate were measured using a Waters HPLC (Waters, Milford, MA) with 410 refractive index detector. The Aminex column (HPX-87H, Bio-Rad, Hercules, CA) was used at 30 °C with 0.3 mL/min of 5 mM sulfuric acid as mobile phase.

2.2.3. Physiological parameters determination

The substrate uptake rate and production secretion rate in batch culture are constant during exponential phase. The substrate uptake rate and product secretion rate are defined as the coefficient of substrate/product concentration versus biomass divided by the growth rate. Acetate is the only product detected under aerobic batch cultivation.

2.2.4. Sample preparation for 2-dimensional NMR analysis

Cells were prepared as described previously (Choudhary et al., 2011). Briefly, cells are centrifuged, washed twice with saline water containing 0.9% NaCl, then hydrolyzed with 6 N hydrochloric acid at 110 °C for 18–24 h. Acids were evaporated, the residue reconstituted in nanopure water and filtered, then lyophilized. Finally, the sample was dissolved in deuterium oxide for NMR analysis.

2.2.5. NMR measurement

2D [^{13}C, ^{1}H] Heteronuclear Single Quantum Correlation (HSQC) spectra were acquired on a Bruker Avance DRX 500 MHz spectrometer at 298 K and processed as described previously (Choudhary et al., 2011; Sriram et al., 2004). Nonoverlapping multiplets on the spectrum were quantified using NMRView (Johnson and Blevins, 1994). Overlapping multiplets (α amino acids) were analyzed using a peak deconvolution software (Choudhary et al., 2011). The amino acids isotopomer abundances measured by 2D HSQC NMR are related to the precursor metabolites by using amino acids biosynthesis pathways as described by Szyperski (Szyperski, 1995). The resulting NMR intensities were used to calculate the isotopomer fractions as shown in Supplementary Table S4.

2.2.6. Metabolic network model for MFA

A network model for *E. coli* metabolism was constructed based on existing literature, Ecocyc database and microarray data (see Table S1). The model includes glucose transport and phosphorylation pathway, Embden–Meyerhof–Parnas pathway, oxidative pentose phosphate branch, non-oxidative pentose phosphate branch, TCA cycle, anaplerotic pathways, metabolite exchange reactions, ED pathways, all amino acids biosynthesis pathways, and several amino acids transamination reactions (Fischer and Sauer, 2003a; Fischer et al., 2004; Sauer et al., 1999; Siddiquee et al., 2004; Toya et al., 2010).

2.2.7. Flux evaluation methodology

Fluxes were quantified using NMR2Flux software developed by Sriram et al. (2004). NMR2Flux employs isotopomer balancing and a global optimization routine to find stoichiometrically feasible fluxes set consistent with experimental measurements. Overall fluxes were estimated by minimizing the chi square error between experimentally measured and simulated isotopomer fractions of amino acid. Errors in evaluated fluxes were estimated from errors in the extracellular fluxes, biomass growth rate, biomass synthesis fluxes, and isotopomer abundances by performing a bootstrap Monte Carlo statistical analysis as explained previously ((Sriram et al., 2004), Supplementary material IV).

2.3. Using OptForce for fatty acid overproduction:

The *i*AF1260 metabolic model of *Escherichia coli* (Feist et al., 2007) was used to perform the simulations with the OptForce procedure (Ranganathan et al., 2010) for overproduction of fatty acids. Metabolic flux data for 35 reactions from the glycolytic, TCA and pentose phosphate pathway was used to define the phenotypic space of a base strain. All simulations were performed under aerobic minimal medium with glucose as the sole carbon source. Glucose minimal conditions were simulated by restricting the glucose uptake rate to 100 mmol gDW^{-1} h^{-1} and the oxygen uptake rate at 200 mmol gDW^{-1} h^{-1}. The lower bound for the remaining exchange fluxes corresponding to the metabolites present in the minimal medium was set to −1000 and the non-growth associated ATP maintenance was fixed at 8.39 mmol gDW^{-1} h^{-1} (Feist et al., 2007). In addition, the biomass flux was fixed at the maximum achievable flux subject to the experimental flux measurements (i.e. 52% of the maximum theoretical). The upper bound for all other reactions was set to 1000 whereas the lower bound was set to zero and −1000 for irreversible and reversible reactions, respectively. All regulatory restrictions were imported from the *i*AF1260 model (Feist et al., 2007) except for the regulatory constraints repressing the β-oxidation pathway under aerobic minimal condition with glucose as the sole carbon source, which was excluded in this study. This is because previous studies have reported on a significant increase in fatty acids production yield upon removal of the β-oxidation pathway (Steen et al., 2010) implying its activity under this condition. It is important to note that even through the *i*AF120 model contains thiolase reactions hydrolyzing fatty-acyl ACPs to fatty acids, the chain specificity of thioesterases is not captured in the model. The phenotypic space of the wild-type strain consistent with stoichiometry/regulation, uptake rates and flux measurements was constructed by successively maximizing and minimizing each reaction flux in the network subject to the network stoichiometry and all of the constraints mentioned above.

Similarly, the flux ranges consistent with a desired overproducing target for fatty acids of specific chain lengths were obtained by iteratively maximizing and minimizing each flux subject to the network stoichiometry, uptake and medium conditions, regulatory constraints and overproduction target. In this study, we imposed a minimum production yield of 90% of the theoretical maximum for all fatty acids of different lengths, while the biomass flux was constrained to be at least 10% of its theoretical maximum. The remaining parameter values were the same as those in the wild-type. OptForce was subsequently used to identify the minimal set of reactions/genes that must be up-/down-regulated or knocked out so as to maximize the formation of targeted fatty acids. OptForce contrasts the maximal range of flux variability between the wild-type strain against the ones consistent with the overproducing phenotype designed to meet a pre-specified yield (i.e., 90%) for hexanoate, octanoate, decanoate, dodecanoate, tetradecanoate and palmitate, respectively. As outlined in earlier efforts, by superimposing the flux ranges one-at-a-time, the fluxes that must depart from the original ranges in the face of overproduction (i.e., $MUST^{U}$, $MUST^{L}$, $MUST^{X}$ sets) are identified. One can extend this classification procedure by considering sums and differences of two fluxes ($MUST^{UU}$, $MUST^{UL}$, $MUST^{LL}$ sets) and arrive at a collective set of flux changes that must happen in the network for overproduction. In this study, we

only considered up to MUST pairs as the available MFA data provided sufficient information to characterize the wild-type strain without having to consider higher order MUST sets. For example, we identified 193 MUST single reactions and 33 reactions participating in MUST doubles reactions (for C_8 fatty acid overproduction) providing a rich set of reaction alternatives to directly engineer. We subsequently extracted the 'minimal' subset(s) of these reactions needed to guarantee the imposed bioengineering objective (i.e., FORCE sets).

In this study we slightly modified the original formulation presented in (Ranganathan et al., 2010) for identifying the FORCE set. In particular, we make use of a max–min bilevel optimization problem to identify alternative sets of k (pre-specified) engineering interventions that maximize the minimum product formation (worst-case scenario) in the network (see Supplementary Text S1). Modeled as a "worst-case" optimization problem, the OptForce procedure identifies metabolic interventions that guarantee an increase in the yield even when metabolic fluxes are allotted so as to directly counteract the desired overproduction. This optimization problem is solved successively, starting with a low number of direct interventions (i.e., $k=1$) and then considering more interventions (by increasing k) until the target yield is achieved. Given that the objective function of the outer problem is maximization of the product formation, manipulations with the highest impact on the product yield are identified first. By increasing the value of k, additional modifications that improve upon the previously identified ones are revealed, thereby providing a way of prioritizing the manipulations based on their impact on the product yield. Binary variables are used here to identify pertinent reactions from the MUST sets whose flux should be increased, decreased, or set to zero (i.e. removed) in order to maximize the minimal product formation yield. Removal of reactions associated with in vivo essential genes based on the KEIO collection (Baba et al., 2006; Feist et al., 2007) in minimal glucose growth medium under aerobic condition, was disallowed even for the ones that were not recognized as essential by the *i*AF1260 model. For example, removal of phosphofructokinase (PFK) and fructose-bisphosphate aldolase (FBA) in the glycolytic pathway (which appear in the MUST sets for C_{12}), and glutamate-5-semialdehyde dehydrogenase (G5SD) or glutamate 5-kinase (GLU5K) reactions in the arginine/proline metabolism, (which appear in the MUST sets for all fatty acids), were prevented. Likewise, the removal of reactions whose corresponding genes have been found to form a synthetic lethal (Suthers et al., 2009) was also disallowed. Examples include phosphoglycerate mutase (PGM) in the glycolytic pathway, transketolase (TKT1 and TKT2) in the Pentose Phosphate Pathway, and aconitase (ACONT) in the TCA cycle. In addition, metabolic interventions in the reaction level inconsistent with gene-level manipulations were avoided in the FORCE sets. For example, the simultaneous up-regulation and down-regulation (or removal) of chain elongation reactions for two fatty acids of different lengths were prevented if they were encoded by the same gene(s). The binary variables corresponding to the reaction interventions that appear in all solutions, as well as those corresponding to trivial solutions (e.g., the up-regulation of the transport reaction corresponding to target product) were also fixed at one and zero respectively to reduce run time. A biomass flux of at least 10% of theoretical maximum was enforced in all OptForce simulations, along with other constraints mentioned before. The use of integer cuts allows for the identification of alternate optimal solutions that can serve as alternate genetic intervention choices. Notably, when the target yield is not achievable with interventions selected only from within the MUST (single and pair) sets we allow for one (or more) intervention (knock out/up/down) from outside the MUST sets by addition of appropriate binary variables and constraints to the max–min optimization formulation. For example, this led to the identification of engineering strategies in the β-oxidation pathway, which did not appear in any of the MUST single or double sets (see Results). The termination criterion for the OptForce procedure was either meeting a production yield of at least 90% of theoretical maximum for each fatty acid, or exceeding the maximum allowable number of reaction interventions (i.e., eight). It is worth noting that the OptForce procedure operates at the reaction level and the set of manipulations at the gene level are subsequently identified manually by using gene–protein-reaction (GPR) associations presented in the model. In principle, we could have run OptForce at the gene level by appending the corresponding GPR constraints in the formulation. However, we have found that it is more instructive to first identify interventions at the reaction level to fathom the reasoning behind the identified interventions.

2.4. Metabolic interventions and fatty acid titer determinations

2.4.1. Fermentation procedure

Each strain was freshly transformed and streaked on LB plate with 100 mg/L ampicillin and incubated at 30 °C overnight. A single colony from the plate was grown in 5 mL M9 medium supplemented with 1.5% glucose and 100 mg/L ampicillin for 16–20 h in orbital shaker at 30 °C and 250 rpm. The pre-culture was then inoculated into 250 mL flasks containing 40 mL M9 medium with 1.5% glucose and 100 mg/L ampicillin. The expression of acyl-ACP thioestersase was induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to final concentration of 1 mM. Samples were taken at 24 h and 48 h for fatty acid and extracellular metabolite analysis.

2.4.2. Fatty acid analysis

Cell cultures were harvested and prepared for fatty acid analysis as described previously (Zhang et al., 2011). Fatty acids in the broth were extracted using chloroform, methylated into methyl esters and recovered using hexane. Tridecanoic acid, pentadecanoic acid and heptadecanoic acid were added as internal standards in all samples before extraction. The fatty acid content was analyzed using an Agilent GC-FID/MS system. The GC system occupies single quadrupole mass spectrometer with an electron impact ionization source and FID detector. The DB-5MS column (30 m, 0.25 mm i.d., 0.25 μm, Agilent) was used to separate the fatty acids into different chain lengths. The oven temperature was initially set at 50 °C for 1 min and raised to 140 °C with 20 °C/min ramping rate. The temperature was then increased to 220 °C with 4 °C/min ramping rate and then finally raised to 280 °C with 15 °C/min ramping rate. Helium was used as the carrier gas with flow rate of 1 mL/min. Interface temperature and ion source temperature was set as 280 °C and 250 °C respectively. EI ionization was set at 0 kV relative to the tuning. Mass spectra were analyzed using the full scan method. Raw MS and FID data was integrated using Chemstation software. Compound peaks were assigned by running standards or referring to the mass fragmentation in the NIST library.

3. Results

3.1. Flux measurements

In vivo metabolic flux analysis, based on the use of ^{13}C-labeled glucose followed by NMR analysis and isotopomer balancing, was used for the estimation of intracellular fluxes as it provides a more comprehensive description of the metabolic network operating under the physiological conditions. Media and temperature conditions for the production of free fatty acids by *E. coli* include

LB rich medium (Zhang et al., 2012b, 2011); (Li et al., 2012) at 30 °C, M9 minimal medium at 37 °C (Zhang et al., 2012a), and MOPS minimal medium at 37 °C (Youngquist et al., 2012). A reduced temperature has been noted to help stabilize strains carrying the plant acyl-ACP thioesterase being unstable (Zhang et al., 2011). Thus, two sets of flux experiments were performed; one for *E. coli* MG1655 in MOPS medium at 37 °C and one for ML103 (MG1655 Δ*fadD*) in minimal M9 medium at 30 °C, both kept under fully aerobic growth in 400 ml batch reactors.

The flux maps for MG1655 under MOPS media and ML103 under M9 media are tabulated in Table S2 and shown in Figs. S1 and S2 in the supplemental material. The flux values are all normalized to 100 mmol $gDW^{-1}\,h^{-1}$. Inspection of the normalized flux data in Table S2 indicates that the flux values are nearly identical for the two experiments. Most of the carbon flux (around 88%) is directed towards the glycolytic pathway, resulting in high activities of the lower glycolytic pathway. Around 10% of the carbon flux channels through pentose phosphate pathway to generate NADPH for reduction requirements. The ED pathway and glyoxylate pathway have negligible fluxes. The anapleurotic pathway of phosphoenolpyruvate (PEP) carboxylase is active, which converts PEP to oxaloacetate (OAA) to refill the OAA pool for biosynthesis. These results are consistent with previous flux experiments showing active PEP carboxylase activity and inactive glyoxylate cycle under glucose aerobic batch culture (Fischer and Sauer, 2003a, 2003b). Acetate kinase is active to convert acetyl-CoA for acetate production. The TCA cycle operates at 45 mmol $gDW^{-1}\,h^{-1}$ (based on 100 mmol $gDW^{-1}\,h^{-1}$) to generate ATP and NAD(P)H for energy and reduction requirements for cell growth. Malic enzyme activity is not significant (Fischer and Sauer, 2003a).

3.2. OptForce results

3.2.1. Targeted pathway

Fig. 1 illustrates the pathways involved in fatty acid biosynthesis in *E. coli*. There are two important fatty acid pathways in *E. coli* metabolism (Fujita et al., 2007; Marrakchi et al., 2002; Schweizer and Hofmann, 2004). Type II or dissociated fatty acid biosynthesis (FAB) pathway involves the ATP-dependent acetyl-CoA carboxylase (encoded by *accABC*) as the first step. Acetyl-CoA is converted into malonyl-CoA which is further converted into malonyl-ACP by the enzyme malonyl-CoA: ACP transacetylase encoded by the gene *fabD*. The initiation of fatty acid biosynthesis starts with the C_4 chain where one mole of acetyl-CoA and one mole of malonyl-ACP synthesize a 4-carbon fatty acid acyl carrier protein (i.e. butyryl ACP). Butyryl ACP further elongates into the 6-carbon chain by recruiting one mole of malonyl-ACP to produce hexanoyl-ACP. This chain elongation step uses one mole of malonyl-ACP per cycle to form even-numbered fatty acid acyl carrier proteins. Fatty acid ACP is converted into a fatty acid by thioesterases in a single step enzymatic conversion. An alternative biosynthesis route for the production of fatty acids that has gained attention recently (Dellomonaco et al., 2011) is reversal of the β-oxidation pathway. While the native use of this pathway is to disassemble longer chain fatty acid ACP into smaller coenzyme-A derivatives, reversal of this pathway may lead to fatty acid synthesis as was recently demonstrated by Dellomonaco et al. (2011) for overproduction of 1-butanol and a number of long chain fatty acids. The calculated maximum theoretical yield for both pathways is similar; however, the type II fatty acid biosynthesis is dependent on the availability of ATP and the specificity of the termination enzyme (i.e., thioesterase) ((Dehesh et al., 1996); (Lennen et al., 2010)). In this paper, we use OptForce for identifying genetic interventions in *E. coli* that result in increased production of free fatty acids using the type II fatty acid pathway.

3.2.2. Identification of MUST sets

Fig. 2 summarizes the set of fluxes in the network that MUST change when overproduction objectives for fatty acids of specific lengths are imposed. Results are presented in Fig. 2 so as to highlight the conservation of MUST changes as the fatty acid chain length increases (Fig. 2a) or decreases (Fig. 2b). For example, in Fig. 2a the additional reactions that enter the MUST set are shown within the growing ellipses as the fatty acid length changes from 6 to 16 carbons. In contrast, in Fig. 2b the reactions added to the MUST set are listed as the fatty acid length decreases from 16 carbons down to 6. This figure thus provides a pictorial view of the conservation patterns of the required changes (i.e., MUST sets) in the metabolic networks as the fatty acid chain length increases or decreases, respectively. For example, increase in the flux for any of the chain elongation reactions 3-oxy-acyl-ACP synthase (3OAS60/80), 3-oxo-acyl-ACP reductase (3OAR40/60/80) and 3-hydroxy-acyl dehydratase (3HAD40/60/80) ensure higher flow of carbon through the fatty acid synthesis pathway, and augment fatty acid production. These chain elongation reactions appear in the MUST sets of fatty acids up to C_{12}, as is shown in the C_{12} ellipse on the right. Even though these reactions do not appear in the (single or double) MUST sets of C_{14} and C_{16} fatty acids, it is likely that they appear in higher order MUST sets (i.e. Must triples, quadruples). Elimination of acetate kinase (ACK) and phosphotransacetylase (PTA) appear in MUST sets of all fatty acids as shown in the C_6 ellipse on the left, as they prevent degradation of pyruvate towards fermentation byproducts. Similarly, elimination of malate dehydrogenase (MDH) and down-regulation of citrate synthase (CS) redirects metabolic flux towards fatty acid synthesis by reducing consumption of acetyl-CoA in the TCA cycle and are universally found for all fatty acid lengths.

In addition to these universal changes, a number of network modifications need to take place for a given chain length or higher (see left panel) or a given chain length or lower (see right panel). For example, up-regulation for enolase (ENO) in glycolytic pathway appear only for C_{10} and longer chains, which is consistent with increased requirements of carbon flux towards fatty acid synthesis for longer chain lengths. Furthermore, up-regulation of pentose phosphate (PP) reaction 6-phosphogluconolactonase (PGL) for fatty acids C_{12} and longer, and eliminations of glycolytic pathway reactions phosphofructokinase (PFK) and fructose-bisphosphate aldolase (FBA) are indicative of the increased need of rerouting flux through the PP pathway to produce reducing agents NADPH required in fatty acid chain elongation.

Notably, reactions along the β-oxidation pathway for the C_8 fatty acid were classified in the $MUST^U$ set of reactions only for hexanoic acid. This is because the *i*AF1260 metabolic model does not contain a fatty acid–acyl-ACP hydrolase that can directly convert hexanoyl-ACP into hexanoic acid. Instead, the favored pathway involves chain elongation into the C_8 chain and subsequently, octanoyl-ACP is reduced into hexanoyl-CoA via the β-oxidation pathway. Eventually, hexanoyl-CoA is converted into hexanoic acid by the thioesterase. However, for the longer chain fatty acids (C_8 or higher), the β-oxidation pathway is not the favored synthesis route because the hydrolase that directly converts ACP-bound end products to the corresponding fatty acids exists in the *i*AF1260 model.

Up-regulation of pyruvate dehydrogenase (PDH), which appears in MUST sets of all fatty acids, leads to the production of acetyl-CoA, which is converted to malonyl-ACP (mal-ACP) fueling the chain elongation reactions. In addition, eliminations in the TCA cycle and pathways branching out from glycolysis, such as glycine hydroxymethyltransferase (GHMT), prevent leaking of the glycolytic flux thus ensuring maximum carbon flow towards fatty acid synthesis chain.

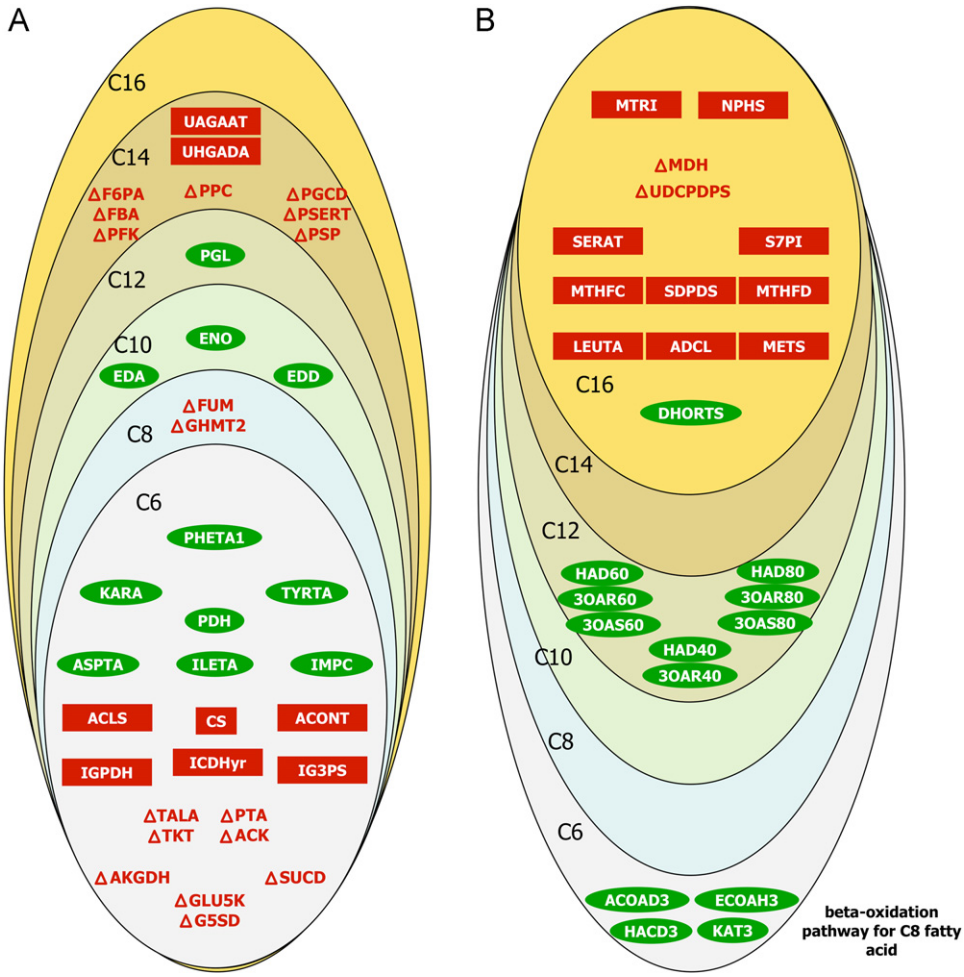

Fig. 2. Venn diagram representing the changes that MUST happen in the network when the overproduction objectives are imposed for fatty acids C_6 through C_{16}. Fluxes that must increase are shown in green boxes whereas the fluxes that decrease are shown in red boxes. The fluxes that must be shut off are shown in red text. (A) The Venn diagram on the left depicts the shared network modification requirements within the C_6 ellipse and additional chain length-specific required changes moving from C_8 to C_{16} fatty acids. Conversely, (B) the Venn diagram on the right shows shared network modifications for all chains lengths within the ellipse for palmitate (C_{16}) and additional chain-specific network changes moving from C_{14} to C_6 acids, respectively.

3.2.3. *Identification of FORCE sets*

Using as candidates the reactions that populate the MUST sets, OptForce max–min optimization formulation (see Methods) is next used to identify minimal sets of engineering modifications for each specific fatty acid chain length. As noted earlier, the termination criterion for the OptForce procedure was either meeting a production yield of at least 90% of theoretical maximum or exceeding the maximum allowable number of reaction interventions (i.e., eight). The identified FORCE sets for each fatty acid chain length are discussed in the following.

3.2.3.1. Hexanoic acid. Fig. 3 depicts the genetic engineering strategies for overproducing hexanoic acid identified by OptForce using the MUST sets as candidate interventions. Results for hexanoic acid, as well as for all higher chain acids revealed that no non-zero minimal yield of product could be guaranteed by using only one intervention. By allowing up to two interventions OptForce predicted the up-regulation of any of the reactions of β-oxidation pathway along the C_8 chain (i.e., octanoyl-CoA dehydrogenase (ACOAD3), 3-oxooctanoyl-CoA dehydrogenase (HACD3) or 3-ketoacyl-CoA thiolase (KAT3)) coupled with the removal of any of the β-oxidation reactions along the C_4 chain (i.e., ACOAD, HACD1 or KAT1). The up-regulation of the β-oxidation reactions along C_8 chain by at least two times the maximum achievable wild-type flux (i.e., from 28 to 54 mmol gDW^{-1} h^{-1}) causes degradation of longer-chain fatty acids to hexanoic acid while the elimination of reactions along the C_4 chain prevents further degradation of any hexanoate formed. It is worth noting that most fatty acid degradation steps corresponding to different chain-lengths in the β-oxidation pathway are encoded by the same gene(s). For example, *fad*E encodes the acyl-CoA dehydrogenase (ACOAD) reactions for all chain lengths while *fad*A does the same for all thiolases (KAT). It is therefore impossible to simultaneously up-regulate the degradation step for a higher chain length and down-regulate (or knock-out) the one for a shorter chain using interventions at the gene level. However, the enzyme catalyzing reaction ACOAD1 (EC 1.3.8.1) differs from the ones catalyzing the same reaction in longer chain acids (EC 1.3.99.3) thereby providing a feasible route for an independent manipulation. OptForce predicted that the aforementioned two interventions would be enough to achieve a theoretical yield of 90% for hexanoic acid and hence, we did not explore additional genetic manipulations.

3.2.3.2. Octanoic and decanoic acid. Metabolic interventions predicted by OptForce for octanoic (C_8) and decanoic (C_{10}) acids are quite similar relying on the strict redirection of carbon flux from glycolytic pathways to fatty acid biosynthesis (see Figs. 4 and 5). OptForce predicts that at least four and five interventions

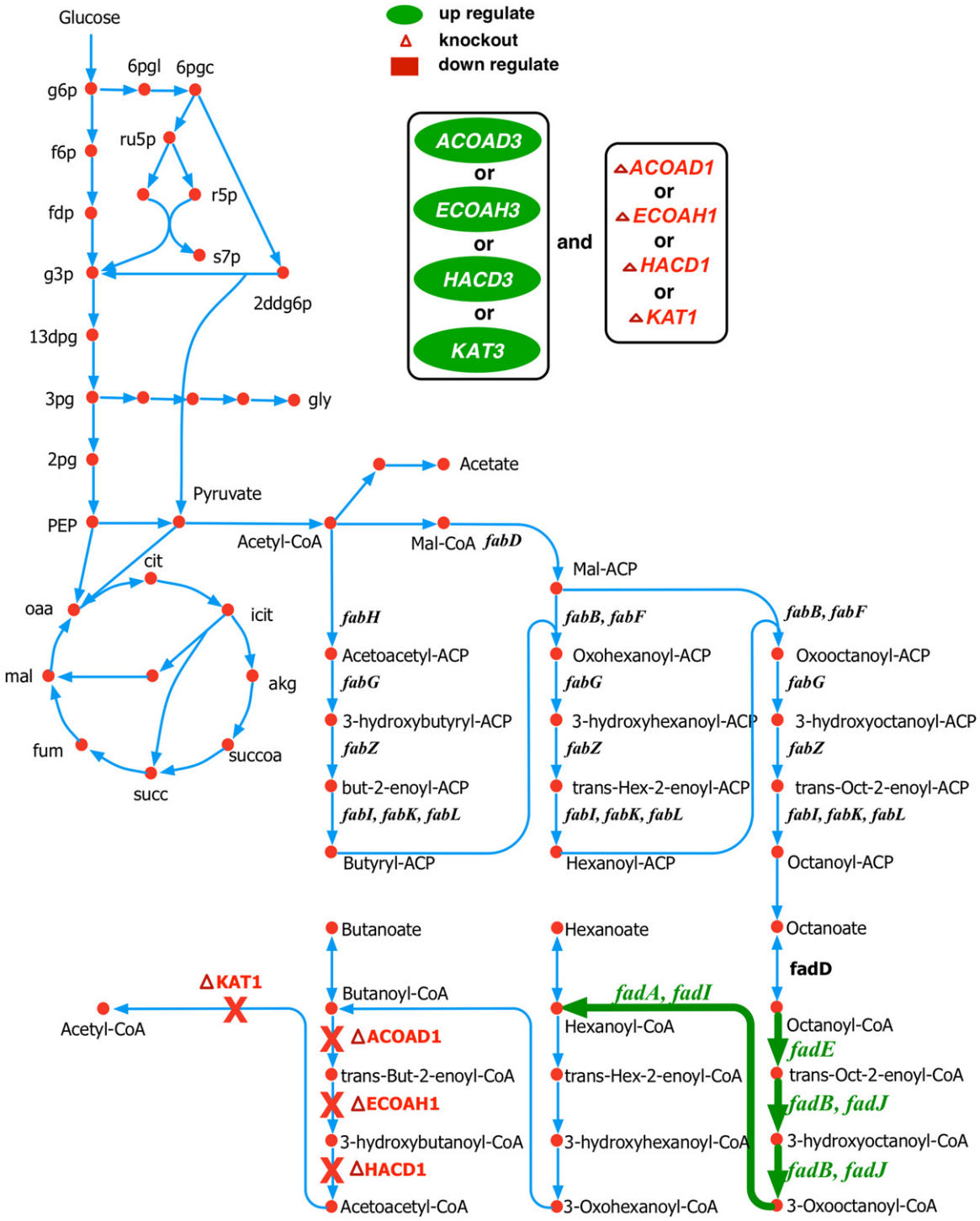

Fig. 3. OptForce interventions for the overproduction of hexanoic acid in *E. coli*.

respectively are required to achieve a theoretical yield of ~90% for both octanoic and decanoic acid. The primary interventions which account for approximately 86% of the yield increase include up-regulation of any of the chain elongation reactions (i.e., 3-oxy-acyl-ACP synthase (3OAS), 3-oxo-acyl-ACP reductase (3OAR) or 3-hydroxy-acyl dehydratase (3HAD)) by at least two times of the maximum achievable flux in the wild-type (i.e., from 28 to 54 mmol $gDW^{-1}\,h^{-1}$) followed by reaction removals in the β-oxidation pathway. OptForce suggests up-regulating any one of the chain elongation reactions along C_8 chain that directly leads to synthesis of octanoate. Reaction removal in the β-oxidation pathway along the C_8 chain (i.e., ACOAD3, ECOAH3i, HACD3 or KAT3) prevents further degradation of the end product. The same holds true for interventions along the C_{10} chain.

OptForce suggests the elimination of fumarase (FUM) in the TCA cycle to maintain a high pool of acetyl-CoA and redirect flux towards the fatty acid elongation chain. This is because the demand of malonyl-ACP, which serves as the primary building block of fatty acids, increases proportionally with the chain length. For example, each molecule of octanoate requires 3 molecules of malonyl-ACP whereas one molecule of decanoate requires four. In addition, OptForce suggests the elimination of acetate kinase (ACK) or

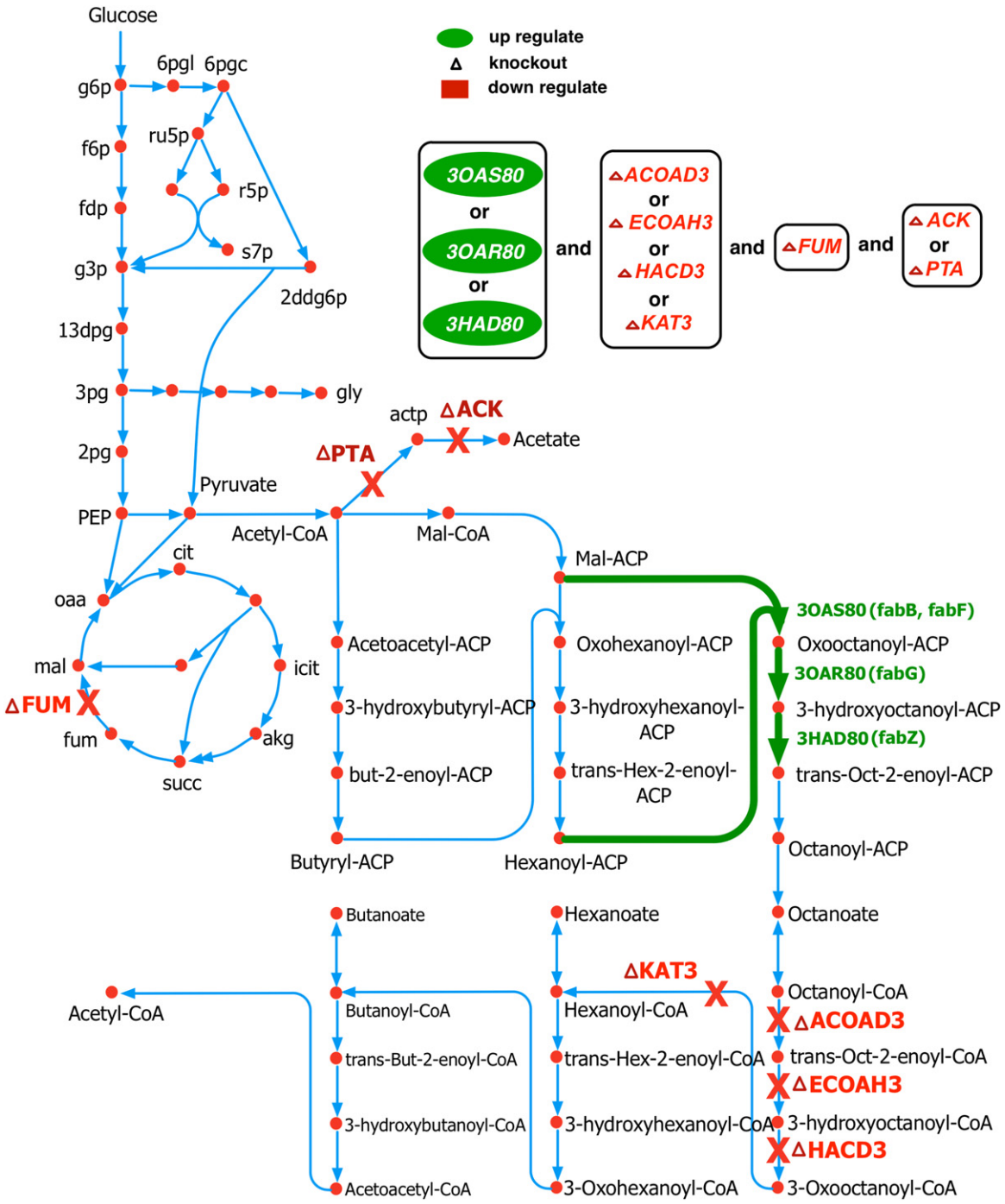

Fig. 4. OptForce interventions for the overproduction of octanoic acid in *E. coli*.

phosphotransacetylase (PTA) possibly to prevent utilization of acetyl-CoA towards production of acetate. Removal of transaldolase (TALA) in the pentose phosphate pathway was also suggested to further redirect glycolytic flux towards decanoate overproduction.

Notably, OptForce does not suggest up-regulation of acetyl-CoA carboxylase as a potential intervention for any of the fatty acids. This is consistent with previous reports where the up-regulation of acetyl-CoA carboxylase (ACCOAC) to increase the pool of malonyl-ACP (Lennen et al., 2010; Lu et al., 2008), did not lead to any significant increase in production of fatty acids. A possible reason for this may be that in the absence of a sink that consumes excess malonyl-CoA (e.g., fatty acid biosynthesis), it is mostly diverted towards biomass component formation.

3.2.3.3. C_{12} and longer chain fatty acids. Figs. 6–8 show the genetic manipulations suggested by OptForce for the overproduction of dodecanoate (C_{12}), tetradecanoate (C_{14}) and palmitate (C_{16}) in *E. coli*. Consistent with the trends observed for octanoate and decanoate, up-regulations of one of the chain elongation reactions for fatty acid pathways (matching the desired chain length) as well as removal of the corresponding β-oxidation pathway are predicted. Interestingly, the specific set of interventions for C_{12} and longer chain length fatty acids includes redirecting glycolytic flux through the oxidative phase of the Entner–Doudoroff (ED) pathway leading to additional NADPH at the expense of ATP production.

Fig. 6 represents the metabolic interventions suggested for C_{12}. Similar to the previous cases, OptForce predicts chain-specific

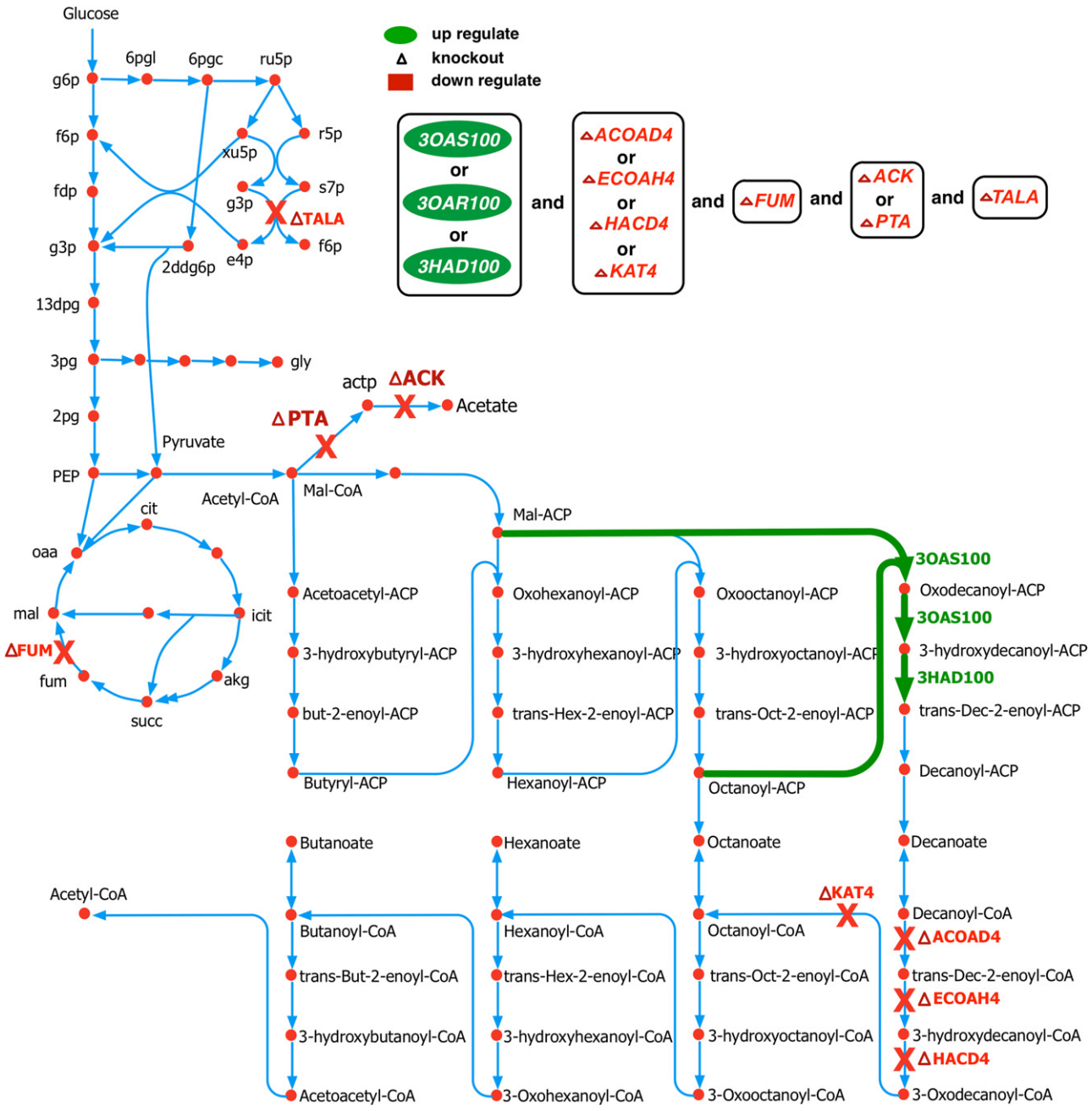

Fig. 5. OptForce interventions for the overproduction of decanoic acid in *E. coli*.

manipulations for reactions in the elongation step of fatty acid biosynthesis and β-oxidation pathways along the C_{12} chain. Interestingly, OptForce also requires an at least eight-fold reduction in the phosphoglycerate mutase (PGM) flux along with the removal of glucose-6-phosphate isomerase (PGI) to bypass the lower and upper glycolytic pathway and instead redirect the metabolic flux towards pyruvate through serine metabolism and Entner–Doudoroff (ED) pathway, respectively. This drastic rewiring of metabolism was suggested by OptForce in order to utilize a less energy efficient route towards production of pyruvate by bypassing the pyruvate kinase (PYK) reaction which generates one mole of ATP and redirecting the metabolic flux through ED pathway, which generates less ATP compared to glycolytic pathway. A possible reason for the preference of a low energy efficient pathway is to arrest the cell growth and channel more metabolic flux towards pyruvate and fatty acid biosynthesis, similar to what has been observed for ethanol production in *Zymomonas mobilis* (Zhang et al., 1995). We computationally explored the validity of this hypothesis by artificially decreasing the energy efficiency of the glycolysis pathway through reducing the stoichiometric coefficient of ATP (and ADP) in phosphoglycerate kinase (PGK). In particular, upon reducing the stoichiometric coefficient of ATP (and ADP) from one to 0.96, we observed a 1.6% increase in the production yield of C_{12} as well as a 1.9% decrease in the maximum biomass formation in the network supporting the put forth hypothesis. Using a less efficient energy pathway leads to the decreased availability of ATP for further conversion of dodecanoate towards tetradecanoate, as manifested by a 95% decrease in tetradecanoate formation. Notably, reducing the stoichiometric coefficient of ATP in PGK beyond 0.96 renders the optimization

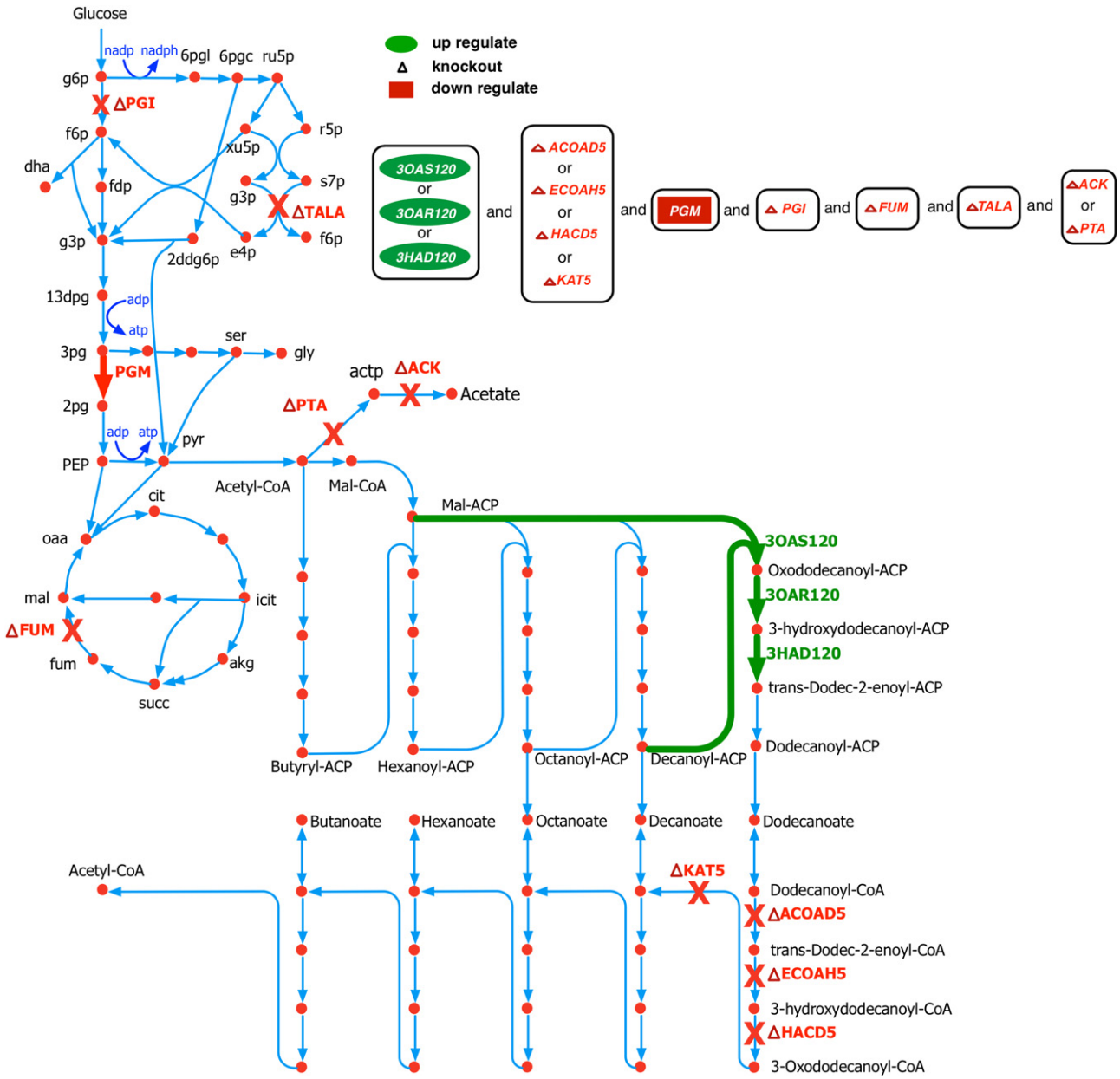

Fig. 6. OptForce interventions for the overproduction of dodecanoic acid in *E. coli*.

problem infeasible, as the imposed constraint on the minimum biomass formation in the network (i.e., 10% of theoretical maximum) cannot be satisfied.

In addition, as fatty acid chain length increases, the NADPH demand for the reduction reactions in chain elongation steps also increases. Hence, OptForce suggests utilization of a more efficient NADPH producing pathway by redirecting the glycolysis flux towards the oxidative phase of PP pathway in order to gain one additional mole of NADPH per mole of glucose (through glucose-6-phosphate dehydrogenase), which could be supplied to 3-oxoacyl-ACP reductase (3OAR) and enoyl-ACP reductase (EAR). A yield of 66% of theoretical maximum was obtained after seven interventions as described above. Interventions for the overproduction of the C_{14} and C_{16} fatty acid (see Figs. 7 and 8) follow the same pattern observed for C_{12}. For example, OptForce predicts the downregulation of PGM flux and removal of PGI to the lower and upper glycolysis, respectively. In addition, OptForce suggests a four-fold reduction of either methenyltetrahydrofolate cyclohydrolase (MTHFC) or methylenetetrahydrofolate dehydrogenase (MTHFD) to reduce metabolic flux from being diverted towards folate metabolism. Additional interventions for C_{16} fatty acid (see Fig. 8), include up-regulation of pyruvate dehydrogenase (PDH) by at least 1.5 times its maximum in the wild-type strain, thus directly enhancing the acetyl-CoA pool for fatty acid production. After seven and eight interventions respectively, OptForce predicted a yield of 65% of theoretical maximum for both tetradecanoate and palmitate. Allowing for further interventions did not lead to any appreciable increase in the guaranteed yield for dodecanoate (C_{12}) or tetradecanoate (C_{14}).

The genetic manipulations required in *E. coli* for the overproduction of palmitate (C_{16} fatty acid) and corresponding impact on the yield are shown in Fig. 9. As seen in the figure, the up-regulation of one of the elongation reactions in the C_{16} chain conjunction with a reaction removal in the β-oxidation pathway, along with redirection of the glycolytic flux leads to an increase in the yield of about 32% of the theoretical maximum. Additional

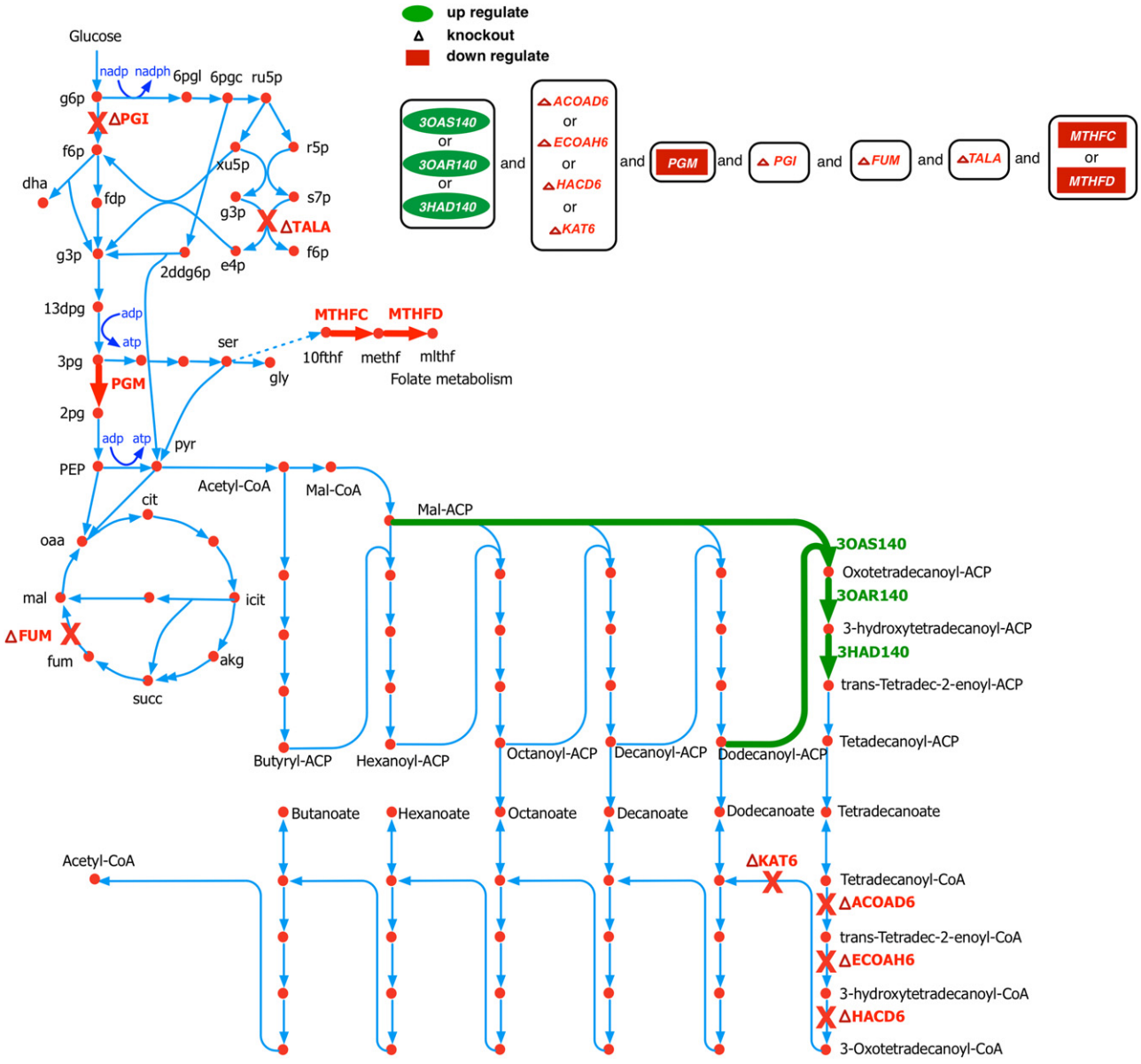

Fig. 7. OptForce interventions for the overproduction of tetradecanoic acid in *E. coli*.

deletions and knock-downs result in improving the yield close to 66% of theoretical maximum. Notably, OptForce suggests that as we move towards longer chain fatty acids, the number of genetic interventions required in central metabolism increases as the carbon flow re-direction becomes more pronounced.

3.3. *Experimental characterization of metabolic interventions*

We chose to test OptForce predictions for the production of medium chain length fatty acids as we had access to plasmids with acyl-ACP thioesterase gene from *Ricinus communis*, which produces a mixture of C_{14} and C_{16} fatty acids. To the best of our knowledge, a thioesterase that strictly produces C_{14} or C_{16} alone has not been identified yet. The first set of prioritized interventions suggested by OptForce for overproduction of C_{14} and C_{16} fatty acid include up-regulation of one of the chain elongation reactions for fatty acid as well as removal of the corresponding β-oxidation pathway (Figs. 7 and 8). We thus implemented these two interventions by overexpresing *fabZ*, which encodes a β-hydroxyacyl-ACP dehydratase, and eliminating the β-oxidation pathway through the deletion of *fadD*.

Strain ML103 (MG1655 Δ*fadD)* with plasmid pXZ18, which carries the gene for the C_{14-16} thioesterase, serves as the reference strain for comparison since it was shown to produce free fatty acid titer and yield similar to those from strain MG1655 pXZ18 in rich media conditions (Li et al., 2012). Since OptForce predictions are based on flux data using defined minimal medium, experiments were performed using minimal M9 medium with 1.5% glucose as shown in Fig. 10. With the overexpression of *fabZ*, the fatty acid titer increased 3.5 fold (from 0.6 g/L for the base strain to 1.7 g/L total fatty acids) after 48 h cultivation, whereas the yield was increased from 0.04 g fatty acid/g glucose for the base strain (~11% maximum theoretical yield) to 0.14 g fatty acid/g glucose (~39% maximum theoretical yield) (Fig. S3). Overexpression of fatty acid elongation reaction pulls the carbon fluxes from acetyl-CoA in the central carbon metabolism to form malonyl-CoA as the precursor for fatty acid synthesis. This "pull" is in addition to the one provided by expression of the

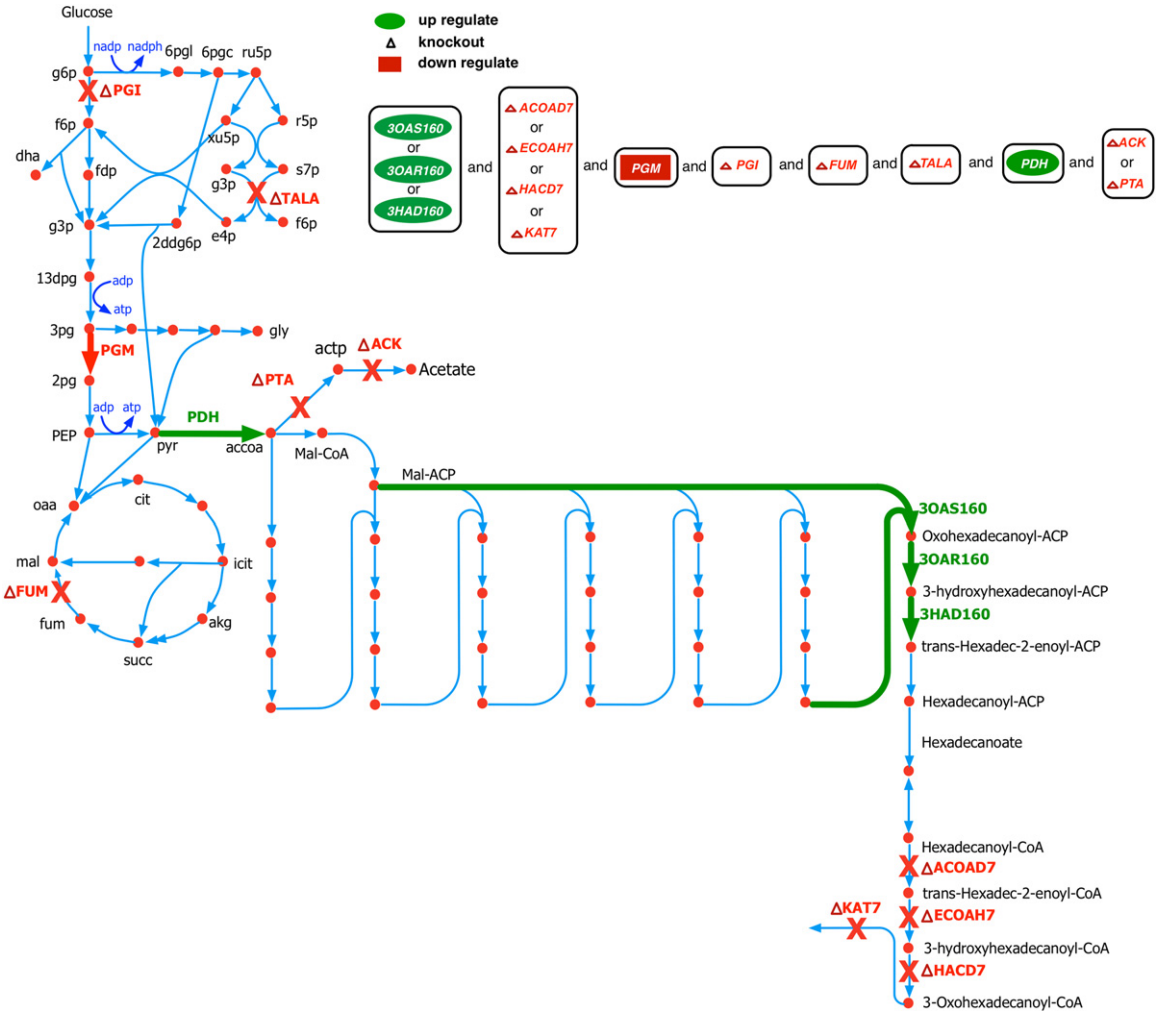

Fig. 8. OptForce interventions for the overproduction of palmitic acid in *E. coli*.

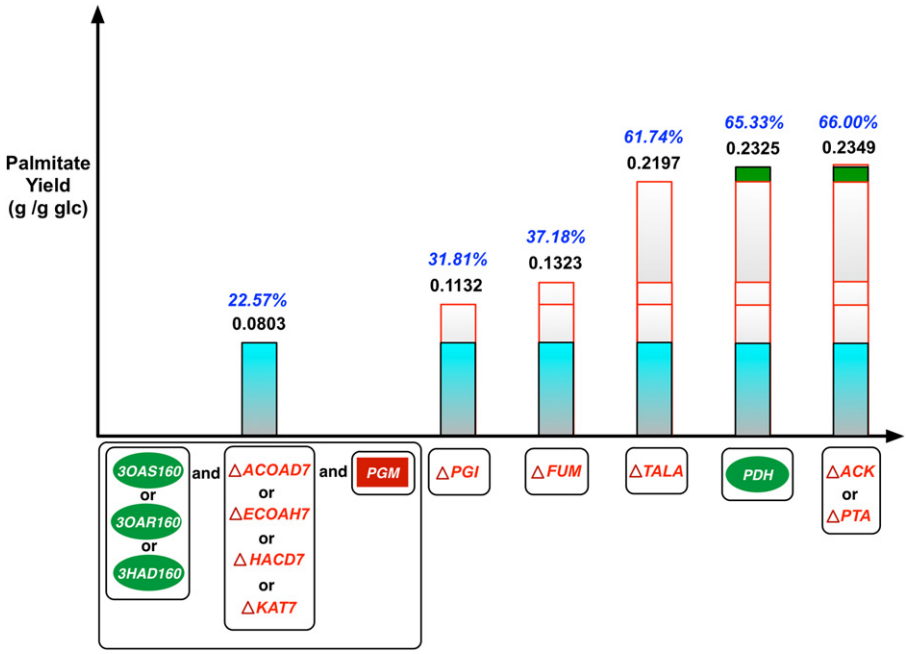

Fig. 9. Impact of each genetic intervention predicted by OptForce on the yield of palmitic acid. The first set of interventions indicates the minimum number of genetic manipulations (i.e., 3 in this case) required to guarantee non-zero yield.

heterologous thioesterase, as the expression of pXZ18 alone already lowered acetate yields from 0.5–0.65 mol acetate/mol glucose in MG1655 and ML103 (Table S3) to ~0.08 mol acetate/mol glucose in ML103 pXZ18 and ML103 pXZ18z (Figs. S4 and S5). It is important to note that OptForce predictions are based upon network stoichiometry alone, and do not involve reaction kinetics. However, the above results suggest that the heterologous thioesterase is not rate limiting in fatty acid production, and that additional interventions in addition to *fabZ* overexpression should help increase the yield.

Notably, another intervention predicted by OptForce includes down regulation of the TCA cycle to maintain high pool of acetyl-CoA and channel the carbon fluxes towards fatty acid synthesis. Nonetheless, OptForce suggests that the fatty acid titer and yield improvement upon this intervention are not as significant as those achievable with overexpression of *fabZ* (see Fig. 9). To test this prediction, we took advantage of an already available strain containing *sucC* deletion in the TCA cycle (MLK163 pXZ18) and it was observed that this intervention increased the fatty acid titer and yield in M9 medium by only 2.4 fold to 1.3 g/L after 48 h, corresponding to a yield of 0.12 g fatty acid/g glucose (see Fig. 10 and Fig. S3), which are lower than those achieved with overexpression of *fabZ* (i.e., 3.5 fold increase) thereby corroborating the OptForce predictions.

The relative composition of the fatty acids produced by the strains ML103 pXZ18 (reference strain), ML103 pXZ18z (*fabZ*$^{++}$) and MLK163 pXZ18 (Δ*sucC*) at 24 and 48 h are shown in Fig. 11, which reveals an abundance of mostly C_{14} and C_{16} straight chain lengths. The fractional composition of C_{14} fatty acid increased over time at the expense of $C_{16:1}$ mono-unsaturated fatty acid. The composition of saturated $C_{16:0}$ fatty acid did not change significantly. Changes in the composition of different fatty acid chain lengths over time could possibly be explained due to the changes in the cellular physiology at different phases of growth (Zhang et al., 2011).

4. Summary and discussion

In this paper, we described computationally derived predictions followed by experimental characterization of strategies for overproducing fatty acids in *E. coli*. Suggested modifications include not only straightforward up-regulations of terminal pathways but also many modifications distant from the fatty acid target that prune away competing pathways, up-regulate pathways to accommodate increased precursor flows or increase the availability of relevant cofactors. Contrary to many existing strategies (Davis et al., 2000; James and Cronan, 2004; Subrahmanyam and Cronan, 1998) that rely on augmenting acetyl-CoA and malonyl-CoA pools, OptForce does not suggest the overexpression of acetyl-CoA carboxylase (*accABC*). As noted earlier, this is to avoid diverting resources towards biomass formation as malonyl-CoA is a key precursor for many biomass constituents. This is in agreement with a recent study (Xu et al., 2011a), that observed experimentally that an increase in the intracellular levels of malonyl-CoA leads to significant cell growth increase. By overexpressing fatty acid enzymes, malonyl-CoA is diverted from biomass formation towards fatty acid biosynthesis. Interestingly, OptForce suggested the up-regulation of one of the four reactions in the β-oxidation pathway for overproducing hexanoate. In the *i*AF1260 *E. coli* metabolic model, acyl-ACP thioesterase enzyme that catalyzes the conversion of hexanoyl-ACP into hexanoic acid is absent. Hence, the desired route to produce hexanoic acid is by elongating the chain further into the C_8 chain and degrading octanoyl-ACP through the β-oxidation cycle.

The chain-dependent nature of the OptForce identified interventions is reflected in the Venn diagram shown in Fig. 12. No universal engineering strategy was predicted for overproduction of all fatty acids, indicating the chain length specificity of each of the strategies. In particular, we observe that the up-regulation of fatty acid reactions is completely chain-specific. For example, for overproducing C_{10} fatty acid the up-regulation of only the C_{10} pathway with elimination in the C_{10} β-oxidation pathway is needed. As we move along in a clock-wise direction (see Fig. 12), genetic manipulations that reduce the activity of TCA cycle and non-oxidative part of the pentose phosphate pathway and increase the carbon flow towards the lower part of glycolysis start to emerge. Interventions for fatty acids of longer chain lengths (i.e. C_{12}, C_{14} and C_{16}) also necessitate diversion of glycolytic flux to the serine metabolism and ED pathway, which are less efficient ATP but more efficient NADPH producing pathways. This redirects flux from cell growth towards satisfying the increased NADPH demand for the reduction steps of the chain elongation pathways. This suggests that upon targeting a fatty acid of higher chain length, a stricter redirection of central metabolic carbon flow towards the precursors becomes progressively more important.

Computational predictions from OptForce were validated for the C_{14}–C_{16} chain length by examining the fatty acid production of a large number of engineered strains carrying a designed medium chain (i.e., C_{14}–C_{16}) thioesterase from *R. communis* in minimal (M9) medium. As mentioned earlier, the common thread from OptForce indicates an up-regulation in the fatty acid chain elongation pathway (except for C_6 fatty acid, which lacks a thiolase to hydrolyze C_6 fatty acyl-ACP) as the intervention of

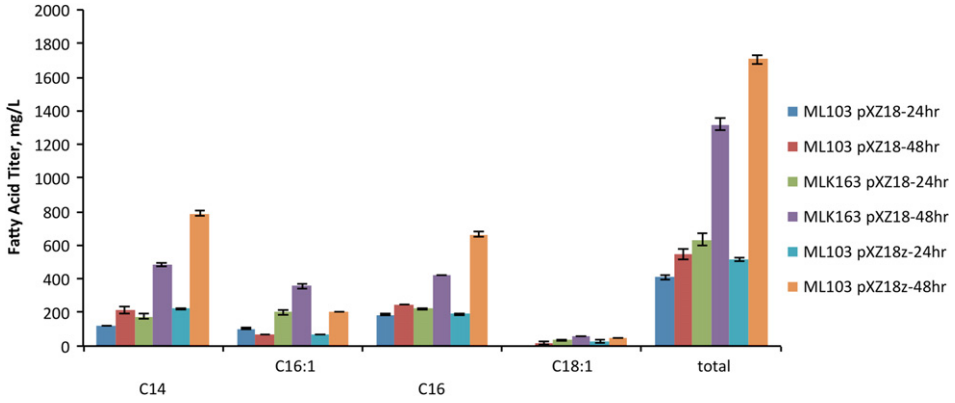

Fig. 10. Accumulation of free fatty acids by ML103 pXZ18 (ΔfadD), MLK163 pXZ18 (ΔfadD, ΔsucC) and ML103 pXZ18z (ΔfadD, fabZ$^+$). The strains were grown in shake flasks in M9 minimal medium with 1.5% glucose at 30 °C and 250 rpm, and sampled at 24 h and 48 h. Error bars represent standard deviation of triplicate cultures.

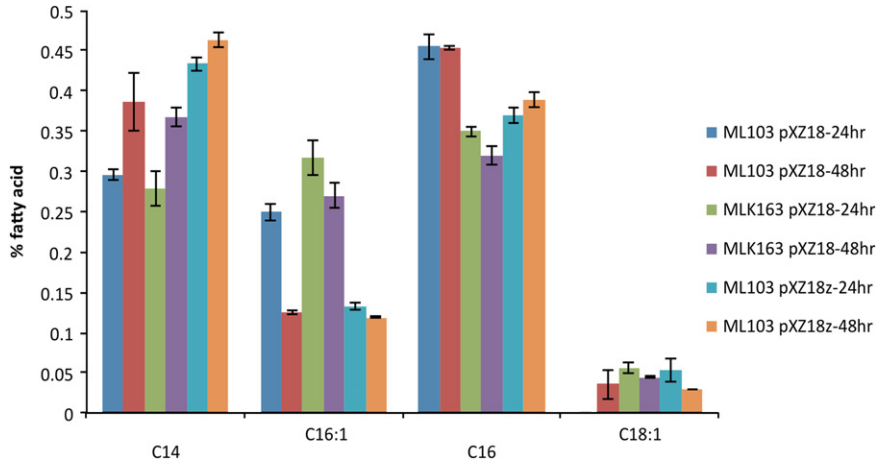

Fig. 11. Free fatty acid composition of ML103 pXZ18(ΔfadD), MLK163 pXZ18 (ΔfadD, ΔsucC) and ML103 pXZ18z (ΔfadD, fabZ$^+$) at 24 h and 48 h. The strains were grown in shake flasks in M9 minimal medium with 1.5% glucose at 30 °C and 250 rpm, and sampled at 24 h and 48 h. Error bars represent standard deviation of triplicate cultures.

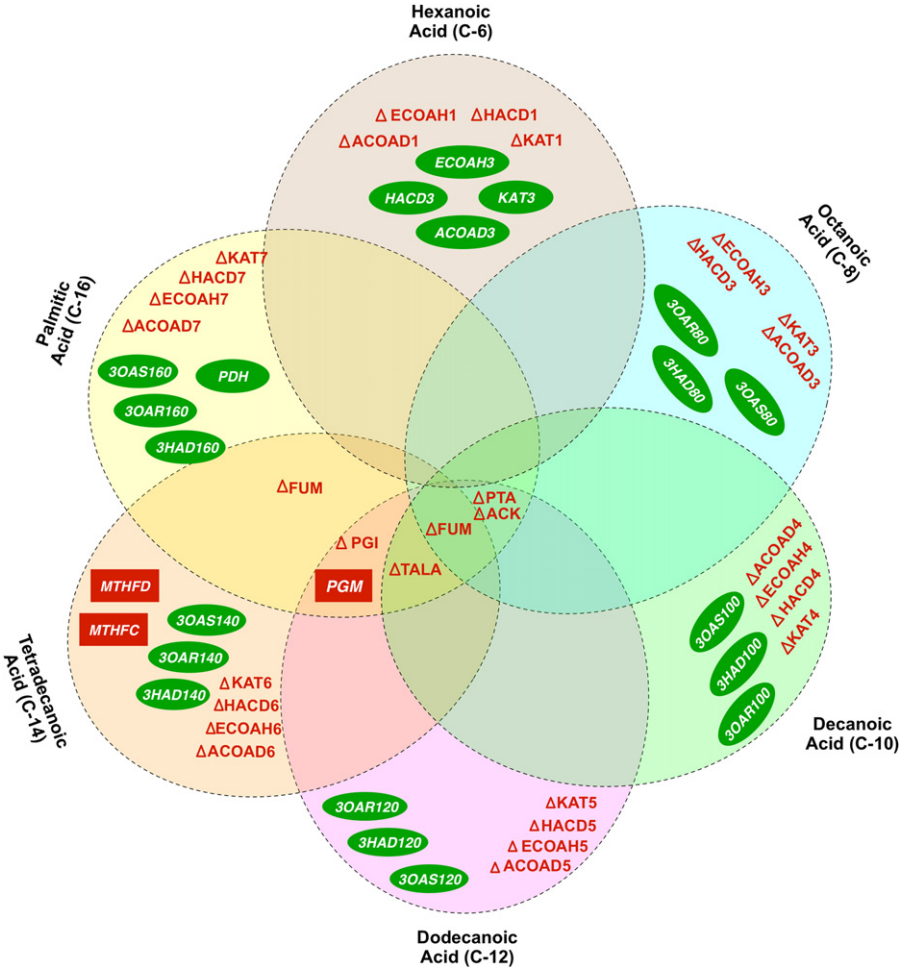

Fig. 12. Venn diagram representing the shared genetic interventions predicted by OptForce for fatty acids of chain length C_6–C_{16}.

primal importance in the overproduction of fatty acids. Transcriptional and post-transcriptional control in *Escherichia coli* tightly regulates the metabolism of fatty acid biosynthesis, making it difficult to decide on specific genetic manipulations. OptForce can only predict the enzymatic steps which require alteration, but cannot provide any inference about regulatory genes. Therefore, several host strains and plasmids were constructed to test our current understanding of regulation in the fatty acid biosynthesis. In accordance with the OptForce prioritization of interventions, *fabZ* and *fadD* were upregulated and deleted, respectively (in a strain carrying upregulated $C_{14–16}$ Acyl-ACP thioesterase) to arrive at a strain that produces 1.7 g/L of $C_{14–16}$ fatty acids and 0.14 g fatty acid/g glucose (~39% maximum theoretical yield) in M9 medium.

We cannot, however, rule out the possibility that other manipulations may increase fatty acid production further. Independent from this work, San and co-workers (2011) recently used a classical "push and pull" concept in metabolic engineering, in which acetyl-CoA supply is enhanced, acetyl-CoA drains are minimized, by-product pathways are eliminated, and product formation pathways are enhanced. A large number of engineered strains carrying a designed medium chain (C_{14-16}) thioesterase, pXZ18, were screened in their ability to increase fatty acid yield and titer in LB medium at 30 °C. The results of their study are summarized in Fig. 13 (San et al., 2011). The increases/decreases shown are in reference to ML 103 (pXZ18), which had a titer of 3.1 g/L and yield of 0.17 g fatty acid/g glucose at 48 h. Although these strains were grown in LB medium and the manipulations were performed independently, the results are in general agreement with OptForce predictions. For example, overexpression of *fabZ* (in a strain carrying *fadD* knockout) leads to the highest yield and titer in rich medium. Manipulation of transcription factors (*fabR* and *fadR*) in fatty acid biosynthesis, the deletion of genes in the TCA cycle (*sucC*, *fumAC* and *gltA*) and the deletion of genes in glycolytic pathway (*glk*, *ptsG*, *pfkA* and *pykF*) also improve fatty acid yield in rich media, but not as much as that obtained from *fabZ* overexpression (San et al., 2011). Note that the order of improvements in fatty acid titer was: $fabZ^{++} > fadR^{++} > \Delta sucC > \Delta fabR > \Delta glk > \Delta pykF > \Delta fumAC$. This independent classical genetic intervention study not only reinforces OptForce predictions to up-regulate fatty acid biosynthesis, downregulate TCA cycle and redirect glycolysis flux towards fatty acid production, but also closely emulates the prioritization of interventions suggested by OptForce.

Even though both *fabA* and *fabZ* are genes responsible for dehydration of *β*-hydroxyacyl-acyl carrier protein (ACP) in *E. coli,* overexpression of *fabA* gene in fatty acid elongation cycle led to a decrease in fatty acid titer and yield (Fig. 13) (San et al., 2011). Similarly, Yu et al. (2011) reported on a decrease and no changes in fatty acid production upon overexpression of *fabF* and *fabZ*, respectively, however, they observed a 50% increase upon the collective upregulation of *fabZ, fabI* and *fabG* in a strain carrying *fadE* deletion, *tesA* overexpression and heterogonous overexpression of a medium-chain specific thioesterase. These reports contradict with the observation in this study that C16 production increases upon upregulation of only *fabZ*. These conflicting results may be explained by the high complexity of fatty acid synthesis regulation. Indirect up-regulation Yu et al of fatty acid chain elongation reactions by deletion of *fabR* and overexpression of *fadR* was shown to increase fatty acid titer and yield (San et al., 2011). *FabR* and *fadR* transcription factors regulate fatty acid biosynthesis in *E. coli* simultaneously. *FabR* represses the expression of the fatty acid synthesis gene *fabB* and *fabA*. On the other hand, *fadR* acts as a repressor to regulate fatty acid degradation and it also activates *fabA* and *fabB* (Campbell and Cronan, 2001). Even though OptForce does not incorporate gene regulatory networks in its framework, the overall idea of upregulating fatty acid chain elongation steps can pinpoint some target transcription factor to be engineered.

Removal of fumarase reaction (FUM) suggested by OptForce was tested independently by San et al. (2011) using a strain carrying *fumAC* knockout, where only small increase of fatty acid titer and yield was achieved, which is in agreement with OptForce prioritization of interventions. An alternative for fumarase knockout to downregulate TCA cycle flux and redirect carbon flux to malonyl-CoA pool was *sucC* knockout which was also tested by San et al. (2011). It was observed that *sucC* deletion outperforms deletion of *fumAC*, however the reason for this is not clear yet. Overall, downregulation of TCA cycle is proven to be an effective strategy to reroute carbon fluxes towards fatty acid synthesis.

OptForce also suggests downregulation of phosphoglycerate mutase (PGM) flux and removal of glucose-6-phosphate isomerase (PGI) reaction in the glycolytic pathway to redirect carbon fluxes through pentose phosphate pathway for medium chain fatty acid production. Even though these interventions were not performed experimentally in this study, previous efforts have already indicated the impact of these removals on improving fatty acids production yield. For example, San et al. (2011) manipulated upper and lower glycolysis with deletion of *glk, ptsG, pfkA* and *pykF* (Fig. 13). More than 20% of improvement in fatty acid yield

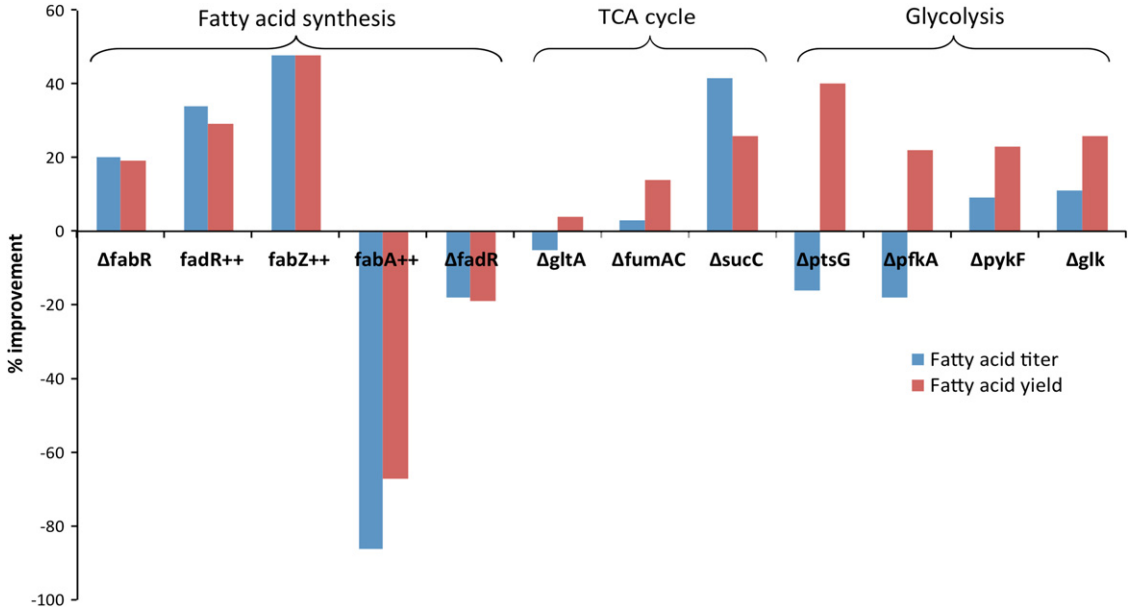

Fig. 13. Effect of different genetic modifications on the improvement of fatty acid titer and yield reported by San *et al* (2011). All the genetic modifications were carried out in *E. coli* strain ML103 (Δ*fadD*). An acyl-ACP thioesterase (pXZ18) was overexpressed in engineered strains to test the effect of the gene knockout (Δ) or overexpression (++). The strains were cultured in LB media with 1.5% glucose and sampled at 48 h. Fatty acid titer and yield improvement were compared with those of the reference strain ML103. Fatty acid titer and yield for the reference strain ML103 are 3.1 g/L and 0.17 g/g.

was achieved among all the strains. Deletion of *pfkF* and *pykF* genes showed slight improvement in fatty acid titer, whereas deletion of *ptsG* and *pfkA* showed inferior productivity. A possible explanation is the biological burden on the cells causing slower growth and lower glucose uptake rate. However, *R. communis* thioesterase effectively pulls the carbons towards fatty acid synthesis even with lower glucose uptake, leading to improved fatty acid yield.

In another study Steen and coworkers (2010) reported ~0.7g/L and ~1.1g/L free fatty acid titer upon cytosolic expression of *E. coli* thioesterase *tesA* along with deletion of β-oxidation pathway genes *fadD* and *fadE*, respectively. Since fatty acid production is highly regulated, Zhang et al. (2012a) subsequently developed a dynamic sensor-regulator system to produce fatty acid-derived products in *E. coli*. Fatty acid/acyl CoA biosensor was engineered based on *fadR* transcription factor and transformed into an *E. coli* strain carrying cytosolic *tesA* thioesterase. This strain was reported to produce 3.8 g/L fatty acid after 3 day cultivation under M9 medium with 2% glucose supplemented with MOPS, mineral and micronutrients. The fatty acid titer was lower in M9 media than in LB media due to nutrient limitation. We anticipate that higher fatty acid production for ML103 pXZ18z (Δ*fadD, fabZ*$^{++}$) can be achieved by using richer medium, higher glucose supplement and longer culturing period. Further efforts can explore incorporating fatty acid/acyl CoA biosensor (engineered *fadR*) into *E. coli* strain ML103 pXZ18z (Δ*fadD, fabZ*$^{++}$) to increase fatty acid titer. Interestingly, deletion of *fadD/fadE* and overexpression of *fadR* as biosensor agree with OptForce suggestion to up-regulate fatty acid synthesis and to eliminate *β*-oxidation of fatty acid for higher production.

While many earlier studies are based on expert intuition and the use of rich media, a systematic quantitative approach (i.e., OptForce supplemented with experimental flux data) with the employment of defined minimal media employed in this study provide a paradigm to a shorter turnover for strain development and cost saving from an industrial standpoint.

Authors' contributions

JVS and CDM conceived the project. TWT, JMY and YF designed and performed the experiments and the metabolic flux analysis. SR AC and ARZ designed and performed the OptForce simulations SR, AC, ARZ, TWT, JMY, JVS and CDM analyzed the data and wrote the paper. All authors have read and confirmed the manuscript.

Acknowledgment

This material is based upon work supported by the National Science Foundation under Award no. EEC-0813570 and the DOE Office of Science grant DE-SC10822882 to CDM.

Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.ymben.2012.08.008.

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