Metabolic Engineering 41 (2017) 115–124

Contents lists available at ScienceDirect

Metabolic Engineering

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

A systematic optimization of medium chain fatty acid biosynthesis via the reverse beta-oxidation cycle in *Escherichia coli*

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ARTICLE INFO

Keywords:
β-oxidation reversal
Biofuels
Biochemical
Synthetic biology
Metabolic engineering

ABSTRACT

Medium-chain fatty acids (MCFAs, 6–10 carbons) are valuable precursors to many industrial biofuels and chemicals, recently engineered reversal of the β-oxidation (r-BOX) cycle has been proposed as a potential platform for efficient synthesis of MCFAs. Previous studies have made many exciting achievements on functionally characterizing four core enzymes of this r-BOX cycle. However, the information about bottleneck nodes in this cycle is elusive. Here, a quantitative assessment of the inherent limitations of this cycle was conducted to capitalize on its potential. The selection of the core β-oxidation reversal enzymes in conjunction with acetyl-CoA synthetase endowed the ability to synthesize about 1 g/L MCFAs. Furthermore, a gene dosage experiment was developed to identify two rate-limiting enzymes (acetyl-CoA synthetase and thiolase). The de novo pathway was then separated into two modules at thiolase and MCFA production titer increased to 2.8 g/L after evaluating different construct environments. Additionally, the metabolism of host organism was reprogrammed to the desired biochemical product by the clustered regularly interspaced short palindromic repeats interference system, resulted in a final MCFA production of 3.8 g/L. These findings described here identified the inherent limitations of r-BOX cycle and further unleashed the lipogenic potential of this cycle, thus paving the way for the development of a bacterial platform for microbial production of high-value oleo-chemicals from low-value carbons in a sustainable and environmentally friendly manner.

1. Introduction

Concerns regarding global energy demand, crude oil depletion and climate change have stimulated increasing efforts to develop renewable chemicals and fuels using carbohydrates as the feedstock. Exploring diverse microbial pathways based on metabolic engineering and synthetic biology frameworks are a promising alternative to petroleum-based chemical feedstock (Xu et al., 2016). Among all the biofuel molecules, fatty acids exhibit high volumetric energy density, with closely similar physicochemical properties to petroleum-based fuels (Xu et al., 2013). Furthermore, fatty acids are precursors for the production of a variety of industrially useful oleo-chemicals (Tee et al., 2014). Medium chain-length fatty acids are of particular interest due to lower freezing/cloud point and higher carbon-conversion yield associating with their shorter chain lengths (Xu et al., 2016).

Escherichia coli is an attractive host organism for producing fatty acids as it has fast replication rates, could grow on a variety of carbon sources and be genetically manipulated (Sherkhanov et al., 2014). This bacterial type II fatty acid synthesis (FAB) pathway probably has been most widely engineered and been harnessed for production of free fatty acids, alcohols, esters, and alkanes. However, only high concentration of long-chain (C_{14}–C_{18}) fatty acid production have been demonstrated (8.6 g/L) (Xu et al., 2013) because of both the natural abundance of long-chain acyl-ACPs and thioesterase-mediated depletion of the long chain acyl-ACP pool, as long chain acyl-ACPs exert a feedback-inhibition effect on upstream enzymes in fatty acid synthesis (Torella et al., 2013). Although thioesterases with substrate specificity for medium chain acyl-ACPs could be expressed to produce MCFAs, yields are largely lower than for long-chain fatty acids (240 mg/L), as they are derived from low abundance acyl-ACPs. Thus, tailoring chain-length specificity in microbial fatty acid production is highly desirable.

Compared to the FAB pathway using both acetyl-CoA and malonyl-ACP as starter units and malonyl-ACP as the extender unit (Kim et al., 2016), a recently engineered reversal of the β-oxidation (r-BOX) cycle exhibits great promise as a metabolic platform for synthesizing fatty acids of varying functionalities and carbon lengths (Fig. 1). In contrast to the FAB pathway, this r-BOX cycle functions with coenzyme A intermediates and directly utilizes acetyl-CoA for acyl-CoA chain

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http://dx.doi.org/10.1016/j.ymben.2017.03.012
Received 13 December 2016; Received in revised form 15 February 2017; Accepted 30 March 2017
Available online 07 April 2017

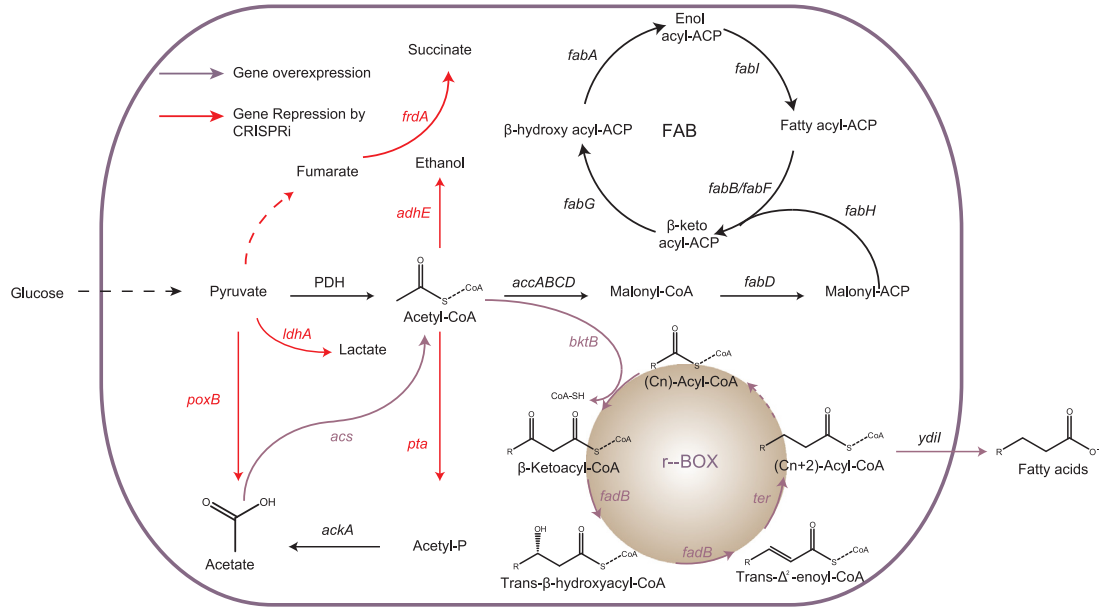

Fig. 1. Synthesis of medium chain fatty acids from D-glucose through the engineered reversal of the β-oxidation cycle. The use of core enzymes of the β-oxidation reversal (thiolase (*bktB*), 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (*fadB*), trans-enoyl-CoA reductase (*ter*), thioesterase (*ydiI*)) in conjunction with acetyl-CoA synthetase (*acs*) enables efficient synthesis of medium chain fatty acids. Genes selected for repressing (*frdA*, *adhE*, *ldhA*, *poxB*, *pta*) are shown in red, while the metabolic pathway that perform the biosynthesis of medium chain fatty acids (*acs*, *bktB*, *fadB*, *ter*, *ydiI*) is shown in purple. FAB and r-BOX indicate the bacterial type II fatty acid synthesis pathway and engineered reversal of the β-oxidation cycle, respectively. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article).

elongations, which supports product synthesis at maximum energy and carbon efficiency (Clomburg et al., 2012; Dellomonaco et al., 2011).

The original implementation of the r-BOX cycle required system-level and complex modifications to global regulators (Dellomonaco et al., 2011), preventing the transfer of this engineered pathways to other host organisms. To overcome this limitation, a synthetic biology strategy was used to functionally characterize the four core enzymes of r-BOX cycle (i.e. thiolases (*atoB* and *fadA*), 3-hydroxyacyl-CoA dehydrogenase (*fadB*), enoyl-CoA hydratase (*fadB*), trans-enoyl-CoA reductase (*ter* and *fabI*)), this enabled constructing a fully transferable and synthetic system for the core components of a one-turn reversal of the β-oxidation cycle (3.4 g/L of butyrate level) (Clomburg et al., 2012; Vick et al., 2015). Further study demonstrated that selection of a key thiolase (*bktB*) and thioesterase (*ydiI*) combining with previously established core enzymes enabled the operation of multiple turns of a reversal of the β-oxidation cycle and generated 1.1 g/L of C_6–C_{10} carboxylic acids (Clomburg et al., 2015). Titers of C_6–C_{10} carboxylic acids were further improved to 1.3 g/L with the use of *tesA*' as the thioesterase termination pathway (Kim et al., 2015).

While this r-BOX cycle has been proven to be advantageous for MCFA production, further developing this platform presents several restrictions. For example, despite many exciting achievements accomplished on identifying four core enzymes of this r-BOX cycle (Clomburg et al., 2015, 2012), the de novo pathway was elusive. More importantly, the information about pathway bottleneck enzymes is limited and remains to be further explored. Pathway imbalances often lead to accumulation of toxic metabolic intermediates and metabolic burden on the host, which result in suboptimal product titers (Biggs et al., 2014; Lu et al., 2015).

Here, in order to fully capitalize on the biosynthetic potential of r-BOX cycle, de novo MCFA synthetic pathway was constructed and a quantitative assessment of the inherent limitations of this pathway was conducted. Two rate-limiting enzymes (*acs* and *bktB*) were identified and the de novo pathway was then separated into two modules to alleviate these two pathway bottlenecks. Additionally, the metabolism of host organism was reprogrammed by clustered regularly interspaced short palindromic repeats interference (CRISPRi) system and a final MCFA titer of 3.8 g/L was observed, representing the highest titers of this chain length distribution reported to date. These results demonstrate that the reversed fatty acid β-oxidation cycle could be further optimized to offer an efficient metabolic pathway for MCFA biosynthesis using a modular r-BOX pathway approach. This modular pathway framework developed here makes r-BOX cycle more easily transferrable to the host of choice for efficient fuel or chemical production without a need for chromosomal mutations.

2. Materials and methods

2.1. Strains, plasmids and general techniques

E. coli JM109 was employed for constructing plasmids. *E. coli* BL21 (DE3) was employed for expressing heterologous pathways and producing fatty acids. Streptomycin (40 μg/mL), ampicillin (100 μg/mL), kanamycin (40 μg/mL) and chloramphenicol (20 μg/mL) were added to maintain the existing of the compatible vectors pCDFDuet-1, pETDuet-1, pRSFDuet-1 and pACYCDuet-1, which were purchased from Novagen (Darmstadt, Germany). DNA ligase and restriction enzymes were purchased from Novagen (Darmstadt, Germany). Thiolase (*bktB*) from *Ralstonia eutropha* (Kim et al., 2015), trans-enoyl-CoA reductase (*ter*) from *Euglena gracilis* (Clomburg et al., 2015, 2012) were codon-optimized for *E. coli* expression (http://www.jcat.de/) and synthesized by GenScript (Nanjing, China). DNA sequences of synthesized genes are showed in the Supplementary material. The absorbance at 600 nm (OD_{600}) was measured by a UV/vis spectrophotometer (UVmini-1240, Shimadzu, Kyoto, Japan).

2.2. Heterologous pathway construction and assembly

Primers and plasmids used in this study are provided in Tables S1 and S2, respectively. All constructed plasmids were verified by both colony PCR and Sanger sequencing. Plasmid constructs and further information are described in Supplementary materials.

2.3. Construction of CRISPRi system to repress target genes

A catalytically dead Cas9 mutant (*dCas9*) and the sgRNA chimera