Engineering *Escherichia coli* for the synthesis of short- and medium-chain α,β -unsaturated carboxylic acids

Seohyoung Kim^a, Seokjung Cheong^a, and Ramon Gonzalez^{a,b}

^a Department of Chemical and Biomolecular Engineering, Rice University, Houston, TX, USA

^b Department of Bioengineering, Rice University, Houston, TX, USA

[#] To whom correspondence should be addressed:

Department of Chemical and Biomolecular Engineering,

Rice University, 6100 Main Street, MS-362,

Houston, TX 77005, USA.

Phone: (713)-348-4893, Fax: (713) 348-5478

email: Ramon.Gonzalez@rice.edu

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Abstract

Concerns over sustained availability of fossil resources along with environmental impact of their use have stimulated the development of alternative methods for fuel and chemical production from renewable resources. In this work, we present a new approach to produce α,β -unsaturated carboxylic acids (α,β -UCAs) using an engineered reversal of the β -oxidation (r-BOX) cycle. To increase the availability of both acyl-CoAs and enoyl-CoAs for α,β -UCA production, we use an engineered *Escherichia coli* strain devoid of mixed-acid fermentation pathways and known thioesterases. Core genes for r-BOX such as thiolase, hydroxyacyl-CoA dehydrogenase, enoyl-CoA hydratase, and enoyl-CoA reductase were chromosomally overexpressed under the control of a cumate inducible phage promoter. Native *E. coli* thioesterase YdiI was used as the cycle-terminating enzyme, as it was found to have not only the ability to efficiently convert trans-enoyl-CoAs to the corresponding α,β -UCAs, but also a very low catalytic efficiency on acetyl-CoA, the primer and extender unit for the r-BOX pathway. Coupling of r-BOX with YdiI led to crotonic acid production at titers reaching 1.5 g/L in flask cultures and 3.2 g/L in a controlled bioreactor. The engineered r-BOX pathway was also used to achieve for the first time the production of 2-hexenoic acid, 2-octenoic acid, and 2-decenoic acid at a final titer of 0.2 g/L. The superior nature of the engineered pathway was further validated through the use of *in silico* metabolic flux analysis, which showed the ability of r-BOX to support growth-coupled production of α,β -UCAs with a higher ATP efficiency than the widely used fatty acid biosynthesis pathway. Taken together, our findings suggest that r-BOX could be an ideal platform to implement the biological production of α,β -UCAs.

1. Introduction

Long-term use of fossil resources poses a number of challenges, including their limited availability and the environmental issues associated with their exploitation such as global warming and air, soil and ocean pollution (Fortman et al., 2008; Kerr, 2007). To cope with these challenges, many technologies have been developed to use renewable resources as alternative feedstocks (Clomburg and Gonzalez, 2013; Vickers et al., 2012). In the energy sector, biological production of hydrogen (Oh et al., 2011), ethanol (Lennartsson et al., 2014) and hydrocarbons (Choi and Lee, 2013; Kallio et al., 2014) from renewables are attracting a lot of interest, while in the chemical sector, 1,3-PDO (Zeng and Sabra, 2011), 3-hydroxypropionic acid (Kumar et al., 2013) and lactic acid (Maki-Arvela et al., 2014) are being commercialized.

Among chemical products of interest, α,β -unsaturated carboxylic acids (α,β -UCAs) are widely used in organic synthesis both as intermediates and final products (Li and Tochtrop, 2014). Owing to their application in food, polymer, perfume and medicine industry, they are synthesized on a commercial scale (Pino and Braca, 1977; Schulz et al., 2000). Efforts have been made to transform fossil-based production of α,β -UCAs into more sustainable platforms. For example, a thermochemical method involving isothermal degradation of biologically-generated poly (3-hydroxybutyrate) (PHB) by a *Cupriavidus necator* strain to produce crotonic acid at a 60% conversion yield has been recently reported (Mamat et al., 2014). As an alternative, purely biological routes can be developed by using microorganisms such as *Escherichia coli*. This bacterium has a dissociated fatty acid biosynthesis (FAB) pathway that consists of four steps using both acetyl-CoA and malonyl-ACP as starter units and malonyl-ACP as the extender unit (Magnuson et al., 1993). Each turn of the FAB cycle adds two carbon atoms to a growing acyl chain. When the acyl chain

reaches the 3-hydroxydecanoyl-ACP intermediate, a double bond is introduced by 3-hydroxydecanoyl-ACP dehydratase/isomerase yielding a mixture of trans-2-decenoyl-ACP and cis-3-decenoyl-ACP (White et al., 2005). While the former is subsequently converted to acyl-ACPs by enoyl-ACP reductase as in the standard elongation cycle, the latter is condensed by 3-ketoacyl-ACP synthases with preserved double bond and elongated to unsaturated fatty acids, such as palmitoleate and cis-vaccenate, the only unsaturated fatty acids synthesized by *E. coli* (Cronan, Jr. and Rock, 2013).

Since enoyl-ACPs are one of the acyl-ACP intermediates of the FAB pathway, hydrolysis of their thioester bond can in principle lead to the production of α - β -unsaturated carboxylic acids (α , β -UCAs). For example, Liu et al. (Liu et al., 2015) reported a specific thioesterase from *B. thetaiotaomicron* (*bTE*) capable of catalyzing the hydrolysis of crotonyl-ACP (2-butenoyl-ACP) and thus leading to the synthesis of crotonic acid (2-butenic acid). To our knowledge, no other α , β -UCA has been produced by fermentation of non-fatty acid feedstocks.

When compared to the FAB pathway described above, an engineered functional reversal of the β -oxidation cycle (r-BOX) has proven to be advantageous in terms of ATP yield since it does not use malonyl-ACP as extender unit for carbon-chain elongation and hence avoids ATP consumption in its synthesis from acetyl-CoA (Cintolesi et al., 2014). This, in turn, can lead to the development of a more effective bio-catalyst (Dellomonaco et al., 2011). Furthermore, the r-BOX uses coenzyme A as the acyl group carrier, thus facilitating the use of native thioesterases as cycle terminators, which tend to have lower K_m values for acyl-CoA groups than acyl-ACPs (Barnes, 1972; Narasimhan et al., 1986; Spencer et al., 1978).

While we have previously engineered r-BOX as a platform for the synthesis of

unsubstituted and β -functionalized carboxylic acids and alcohols (Dellomonaco et al., 2011; Clomburg et al., 2012; Clomburg et al., 2015), no significant production of α,β -UCAs was reported. In this study, we present a novel biological method for efficiently producing α,β -UCAs that uses a modified version of r-BOX that accumulates trans-enoyl-CoA intermediates and an r-BOX cycle-terminating thioesterase capable of converting trans-enoyl-CoAs to α,β -UCAs while exhibiting a low catalytic efficiency for acetyl-CoA. The engineered platform was used not only to produce crotonic acid at a yield 10 fold higher than previously reported, but also to achieve for the first time the production of 2-hexenoic acid, 2-octenoic acid, and 2-decenoic acid. The superior nature of the engineered platform was further validated through the use of *in silico* metabolic flux analysis.

2. Materials and Methods

2.1. Strains, plasmids and genetic methods

Wild-type *Escherichia coli* MG1655 (Kang et al., 2004) was used as the host for all genetic modifications. Gene knockouts and chromosomal expression constructs were introduced in MG1655 and its derivatives by P1 phage transduction (Miller, 1972). All resulting strains used in this study are listed in Table 1. Single gene knockout mutants from the National BioResource Project (NIG, Japan) were directly used as donors of specific mutations. Creation of constructs for the cumate-controlled chromosomal expression of *atoB*, *fadB*, *fadA*, *bktB* and *egTER* were achieved as previously described (Kim et al., 2015). Plasmid-based gene overexpression was achieved by cloning the desired gene into pCDFDuet-1 (Novagen, Darmstadt, Germany), digested with NcoI and EcoRI, and by utilizing In-Fusion PCR cloning technology (Clontech Laboratories, Inc., Mountain View). Resulting plasmids are shown in Table 1. All molecular biology techniques were performed

with standard methods or following manufacturer protocol. Plates were prepared using LB medium containing 1.5% agar and appropriate antibiotics were included at the following concentrations: ampicillin (100 µg/mL), kanamycin (50 µg/mL), spectinomycin (50 µg/mL) and chloramphenicol (12.5 µg/mL).

2.2. Culture medium and cultivation condition

The minimal medium designed by Neidhardt et al. (Neidhardt et al., 1974), modified with 125 mM MOPS (pH 7.4) and Na₂HPO₄ in place of K₂HPO₄ and supplemented with 20 g/L glycerol or glucose, 10 g/L tryptone, 5 g/L yeast extract, 5 mM calcium pantothenate, 2.78 mM Na₂HPO₄, 5 mM (NH₄)₂SO₄, 30 mM NH₄Cl was used for all fermentations. Antibiotics, inducers (isopropyl β-D-1-thiogalactopyranoside and cumate) and CaCO₃ were included when appropriate. Fermentations were conducted in 25 mL Pyrex Erlenmeyer flasks (narrow mouth/heavy duty rim, Corning Inc.) filled with 15 mL of the above culture medium and sealed with foam plugs filling the necks. A single colony of the desired strain was cultivated overnight (14-16 hours) in LB medium with appropriate antibiotics and used as the inoculum (1%) for all fermentations. After inoculation, flasks were incubated for 48 hours at 37°C and 200 rpm in an NBS C24 Benchtop Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ) and IPTG (10 µM) and/or cumate (100 µM) were added unless otherwise specified. The same inoculation procedure was used for bioreactor operation in a SixFor multi-fermentation system (Infors HT, Bottmingen, Switzerland) equipped with 500ml working volume vessel with control of temperature (37°C), stirrer speed (720rpm), pH (controlled at 7.5 with NaOH and H₂SO₄) and 35g/L glycerol.

2.3. Analytical methods

Optical density was measured at 550 nm in a Thermo Spectronic Genesys 20 (Thermo Scientific, Waltham, MA) and used as an estimate of cell mass (1 O.D.550 nm = 0.34 g dry weight/L). Quantification of glycerol and metabolic products in the culture supernatant was conducted through high-performance liquid chromatography (HPLC) and gas chromatography-flame ionization detection (GC-FID). The concentrations of glycerol, ethanol, and organic acids were determined via ion-exclusion HPLC using a Shimadzu Prominence SIL 20 system (Shimadzu Scientific Instruments, Inc., Columbia, MD) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA) at a flow rate of 0.3 mL min⁻¹ using 30 mM H₂SO₄ mobile phase at column temperature 42°C (Dharmadi and Gonzalez, 2005). Quantification of unsaturated carboxylic acids (C > 6) was conducted via GC-FID analysis using a Varian CP-3800 (Varian Associates, Inc., Palo Alto, CA) equipped with FID and an HP-5 capillary column (0.25 mm internal diameter, 0.25 µm film thickness, 30 m length; Agilent Technologies). Sample preparation was conducted as follows: 2 mL culture supernatant samples were transferred to 5-mL glass vials and 80 µL of 50% H₂SO₄ and 340 µL of 30% NaCl solution were added for pH and ionic strength adjustment, respectively. Heptanoic acid (final concentration 50 mg/L) was added as an internal standard and 2 mL of hexane-MTBE (1:1) was added for extraction. The vials were sealed with Teflon-lined septa (Fisher Scientific, Pittsburg, PA), secured with caps, and vigorously vortexed. The samples were then centrifuged for 2 min at 2375 x g to separate the aqueous and organic layers. After centrifugation, 1.5 mL of the top organic layer was separated from the aqueous layer. The organic layer was transferred to a 5 mL glass vial and dried under a stream of nitrogen. 1 mL of a boron trifluoride-methanol solution was added to the dried extract for derivatization of extracted unsaturated carboxylic acids to fatty acid methyl esters (FAMES). The vials were capped tightly and vortexed and then placed in a heater block

(AccuBlock digital dry bath; LabNew, Woodbridge, NJ) and heated at 60°C for 60 min. The transesterification reaction vials were cooled to room temperature, followed by the addition of 1 mL of water and vigorous vortexing. The vials were centrifuged for 2 min at 2375 x g to separate the aqueous and organic layers. The upper organic phase was collected with a glass syringe and transferred to GC vials (Fisher Scientific, Pittsburg, PA) for GC analysis according to the following method: 1 µL sample injection run in splitless mode using helium as the carrier gas with a flow rate of 1 mL/min. The injector and detector temperature were 220°C and 275°C, respectively. The oven temperature was initially held at 50°C for 3min and then raised to 250°C at 10°C min⁻¹ and held for 3 min. Identification of unsaturated carboxylic acids was performed on an Agilent 7890A GC system, equipped with a 5975C inert XL mass selective detector and an Restek Rxi-5Sil column (0.25mm internal diameter, 0.1 µm film thickness, 30m length) as previously described (Clomburg et al., 2012)

2.4. Preparation of thioesterase YdiI

His-tagged thioesterase YdiI was prepared by amplifying *ydiI* from the genome of *E. coli* MG1655 with primers ydiI-L, 5' - AGGAGATATACCATGATATGGAAACGGAAAATCAC-3' and ydiI-R, 5' - CGCCGAGCTCGAATTCTCACAAAATGGCGGTCGTC-3'. The amplicon was ligated with pCDFDuet digested by EcoRI and then transformed into *E. coli* BL21(DE3). A single colony of the desired strain was cultivated overnight (14-16 hours) in LB medium with appropriate antibiotics and used as the inoculum (1%) for the main cultivation. After inoculation, cells were grown at 37°C, 200 rpm to an optical density 0.4~0.5 at 550nm, then 400 µM of IPTG or 100 µM cumate was added to induce protein expression. Grown cells were harvested,

washed twice with a 9 g/L NaCl solution, and stored as cell pellet at -80°C until it was used. Approximately 1 g (dry weight) of cells were disrupted by B-PER bacterial protein extraction reagent (Fisher Scientific, Pittsburg, PA) supplemented with 300 mM NaCl, 5% glycerol, 20 mM imidazole, 5 mM MgCl₂ and 1 mM tris (hydroxyl propyl) phosphine (THP). Purification of YdiI was achieved by affinity chromatography using a Ni-NTA column kit (Qiagen, Valencia, CA). The supernatant fraction of cell lysate was loaded onto the column and the column was washed twice with 600 µl of washing buffer A: 50 mM of NaH₂PO₄, 300 mM of NaCl₂, 5% glycerol, 100 mM imidazole, 5 mM MgCl₂ and 1 mM THP. Elution of YdiI from the column was conducted by using 500 µl of elution buffer B: 50 mM of NaH₂PO₄, 300 mM of NaCl₂, 5% glycerol, 500 mM imidazole, 5 mM MgCl₂ and 1 mM THP. The eluted YdiI was desalted by Amicon centrifugal desalting apparatus (Merck Millipore, Billerica, MA) with molecular cut-off of 10 kDa and suspended in the assay buffer: 500 mM Tris-HCl (pH 7.5), 200 mM KCl.

2.5. Enzyme assays

Assays for thiolase, 3-hydroxyacyl-CoA dehydrogenase, and thioesterase activity were carried out as described previously (Kim et al., 2015; Vick et al., 2015). Activity of AtoB (thiolase) was monitored by measuring the decrease in acetoacetyl-CoA at 303nm. Activity of FadB (3-hydroxyacyl-CoA dehydrogenase) was measured by monitoring the oxidation rate of NADH at 340nm in the presence of acetoacetyl-CoA. The measurement of YdiI activity (thioesterase) was carried out by monitoring the reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) at 412nm. All assays were conducted at 30°C and absorbance measurements were performed in a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT)

2.6. *In silico* metabolic flux analysis

The *E. coli* genome-scale model GEM iJO1366 was adapted with curations as described previously (Cintolesi et al., 2014). Additionally, the flux of the acyl-CoA dehydrogenase reaction in the synthetic direction (enoyl-CoA + FAD $\rightarrow$ acyl-CoA + FADH₂) was set to zero because this reaction is thermodynamically infeasible in that direction (Dellomonaco et al., 2011). Production pathways of α,β -unsaturated carboxylic acids were simulated as one lumped reaction to exclude any possible interactions of unsaturated carboxylic acid production with lipid biosynthesis and cell growth caused by the presence of intermediates common to both pathways. The equations for the lumped reactions are: $n/2$ acetyl-CoA + $(n-3)$ NADH + $(n/2-2)$ H⁺ $\rightarrow$ C_n trans-2-acid + $(n-3)$ NAD⁺ + $n/2$ CoA for product synthesis through the engineered β -oxidation reversal; $n/2$ acetyl-CoA + $(n/2-1)$ NADPH + $(n/2-1)$ ATP + $(n/2-2)$ NADH + $(n/2-2)$ H⁺ $\rightarrow$ C_n trans-2-acid + $(n/2-1)$ NADP⁺ + $(n/2-2)$ NAD⁺ + $(n/2-1)$ ADP + $(n/2-1)$ Pi + $n/2$ CoA for product synthesis through the fatty acid biosynthesis pathway; $n/2$ acetyl-CoA + $(n/2-1)$ NADPH + $(n/2-2)$ NADH + $(n/2-2)$ H⁺ $\rightarrow$ C_n trans-2-acid + $(n/2-1)$ NADP⁺ + $(n/2-2)$ NAD⁺ + $n/2$ CoA for product synthesis through a hypothetical fatty acid biosynthesis pathway with an ATP-independent acetyl-CoA carboxylase reaction. n is an even positive integer ≥ 4 . The production of α,β -unsaturated carboxylic acids was simulated under fermentative conditions with a glucose consumption rate of 15 mmol/gCDW/h.

3. Results and discussion

3.1. Re-engineering β -oxidation reversal (r-BOX) to produce α , β -unsaturated carboxylic acids

The synthesis of short- ($C_{4:1}$) and medium-chain ($C_{6:1}$, $C_{8:1}$, $C_{10:1}$) α , β -unsaturated carboxylic acids (α , β -UCA) through an engineered reversal of the β -oxidation cycle (r-BOX) requires the use of core r-BOX enzymes along with thioesterase(s) able to convert enoyl-CoAs to the corresponding α , β -UCAs (Figure 1). The following core r-BOX enzymes were considered for this purpose: *Escherichia coli* thiolase (*atoB*, *fadA*), hydroxyacyl-CoA hydrogenases (*fadB*) and enoyl-CoA hydratase (*fadB*), along with *Euglena gracillis* trans-enoyl-CoA reductase (*egTER*) (Clomburg et al., 2012; Kim et al., 2015). As a first step, we focused on the synthesis of crotonic acid, a 4-carbon α , β -UCA that results from the one-turn operation of the r-BOX. When crotonic acid is the target product, the last step of the r-BOX catalyzed by enoyl-CoA reductase becomes a competing reaction. Therefore, a partial r-BOX was re-engineered in *E. coli* to incorporate only a short-chain thiolase (*atoB*), hydroxyacyl-CoA hydrogenase (*fadB*), and enoyl-CoA hydratase (*fadB*) (Figure 1). Genes encoding these enzymes were incorporated into the chromosome of *E. coli* MG1655(DE3) under the control of a cumate inducible phage T5 (CT5) promoter, resulting in strain MG1655(DE3)-*atoB*^{CT5}-*fadB*^{CT5}. *In vitro* enzyme assays were conducted to confirm chromosomal expression of these three core r-BOX enzymes. The crude extract of the engineered strain showed thiolase and hydroxyacyl-CoA hydrogenases activities of 13.8 $\mu\text{mol}/\text{min}/\text{mg}$ and 2.2 $\mu\text{mol}/\text{min}/\text{mg}$,

respectively, while these activities were undetectable in wild type *E. coli* MG1655(DE3).

The above-engineered strain was used as the host to assess the potential of six native thioesterases (TEs: *yciA*, *ydiI*, *ybgC*, *tesA*, *fadM*, and *tesB*) for the production of crotonic acid. Each TE was individually cloned into the pCDFDuet vector and expressed under the control of a T7 promoter (*tesA* was cloned without leading sequence, labeled as *tesA'*). Resulting plasmids were transformed into strain MG1655(DE3)-*atoB*^{CT5}-*fadB*^{CT5} and production of crotonic acid was assessed in shake flask cultures using glycerol MOPS medium fortified with tryptone and yeast extract and with other conditions adapted from our previous report (Kim et al., 2015). Among all tested thioesterases, only YdiI supported the production of crotonic acid (Figure 2A, 0.19 g/L). The identity of crotonic acid as a fermentation product was confirmed by using GC-MS (Figure 2B and 2C). The amount of crotonic acid produced by this strain exceeded the maximum titer reported in the literature (0.16 g/L) using an engineered fatty acid biosynthesis (FAB) pathway (Liu et al., 2015). It is noteworthy that the previously reported crotonic acid titer of 0.16 g/L was achieved after a number of other manipulations, including the use of a FabI inhibitor (triclosan) and deletion of *fadD* (acyl-CoA synthetase) to increase the intracellular levels of enoyl-ACP. These initial results suggest that the engineered r-BOX has a great potential for the production of α,β -UCA.

3.2. Kinetic characterization of YdiI-encoded enoyl-CoA thioesterase activity

While overexpression of YdiI along with the core genes for r-BOX resulted in significant production of crotonic acid, the enoyl-CoA thioesterase activity of this enzyme and corresponding kinetic properties have not been reported in the literature. N-terminal His-tagged YdiI was purified from strain BL21(DE3) carrying a pCDFDuet vector expressing *ydiI*, as described in *Materials and Methods*. Enoyl-CoA thioesterase activity was assayed

using Ellmann's reagent and crotonyl-CoA as substrate at 30°C in the presence of 200 mM potassium chloride (Barnes, 1972).

As shown in Figure 3, the dependence of YdiI activity on crotonyl-CoA is well described by the Michaelis-Menten equation with $K_m = 524 \pm 18 \mu\text{M}$ and $k_{cat} = 0.93 \pm 0.03 \text{ s}^{-1}$, yielding a catalytic efficiency of $1.77 \times 10^3 \text{ M}^{-1} \text{ s}^{-1}$ (Table 2). When assayed on acyl-CoA substrates, YdiI has proven to have broad substrate range and tends to have higher catalytic efficiency (k_{cat}/K_m) for longer chain acyl-CoAs (Chen et al., 2013; Latham et al., 2014) (Table 2), suggesting that it might support the synthesis of longer-chain α,β -UCAs such as 2-hexenoic acid, 2-octenoic acid, and 2-decenoic acid. Also relevant is the observation that when using acetyl-CoA as the substrate, YdiI shows extremely low k_{cat} and high K_m and hence very low catalytic efficiency on acetyl-CoA ($2.8 \times 10^{-6} \text{ M}^{-1} \text{ s}^{-1}$; Table 2). Since r-BOX uses acetyl-CoA as both primer and extender unit (Figure 1), the low thioesterase activity of YdiI on acetyl-CoA would ensure availability of primer and extender units for the operation of the pathway and minimize the synthesis of acetate as a by-product. Taken together, our findings indicate that YdiI is a good choice of thioesterase for the production of α,β -UCAs through r-BOX.

3.3. Improved synthesis of crotonic acid by using a fermentation-deficient strain and appropriate thiolase

Since the engineered r-BOX is initiated by a condensation reaction between two acetyl-CoAs (Figure 1), increasing intracellular levels of this metabolite could lead to enhancing the synthesis of r-BOX products. Carbon fluxes to alcohols and acids in mixed acid fermentation in *E. coli* mainly diverge from pyruvate and acetyl-CoA. Therefore, knocking-out specific genes responsible for the synthesis of these byproducts can result in

increased carbon fluxes towards the r-BOX pathway. Serial deletion of *ldhA*, *poxB*, *pta*, *ahdE* and *frdA* was carried out in *E. coli* MG1655 (DE3)-*atoB*^{CT5}-*fadB*^{CT5}. The resulting strain, named JC01(DE3)-*atoB*^{CT5}-*fadB*^{CT5}, was transformed with pCDFDuet-p1-*ydiI* and its performance evaluated under the same fermentation conditions used for evaluating the impact of thioesterase overexpression (see section 3.1). The results show not only a decrease in the levels of succinate, lactate and ethanol and increase in pyruvate, as expected, but also a 5-fold increase in crotonic acid production, from 0.19 g/L to 0.98 g/L (Figures 4B and 4C). The crotonic acid yield also increased 4.5-fold, from 0.02 to 0.09 g crotonic acid/g glycerol. Cell growth and glycerol consumption were not significantly affected by these genetic manipulations (Figure 4A), and hence product yield and product synthesis rates (specific or volumetric) increased by approximately five fold.

In a previous report, we have shown that the reaction initiating the r-BOX (thiolase-catalyzed condensation of two acetyl-CoAs) can be a crucial control point with a potentially large impact on the overall efficiency of the pathway (Kim et al., 2015). Three native *E. coli* thiolases (AtoB, FadA, YqeF) and BktB from *Ralstonia eutropha* have been reported to catalyze this reversible condensation reaction required for the r-BOX pathway (Clomburg et al., 2012; Kim et al., 2015). When the aforementioned thiolases were evaluated for the production of crotonic acid, the use of BktB resulted in a 50% increase in product synthesis when compared to the use of AtoB (Figure 4). The corresponding strain (JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}/pCDFDute-p1-*ydiI*) produced 1.5 g/L of crotonic acid with a yield of 0.12g crotonic acid/g glycerol in a 48 hour fermentation (Figure 4). It is conceivable that decrease in pH below 5.5 during flask fermentation as shown in Figure 4A may have detrimental effect on performance of microbial chemical production. Hence, in order to further increase crotonate titer, strain JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}/pCDFDute-p1-*ydiI* was cultivated in a bioreactor maintaining the pH at 7.5, which yielded 3.2 g/L of crotonic acid with a yield of 0.10 g

crotonic acid/g glycerol in a 65 hour fermentation.

3.4. Synthesis of medium-chain α,β -unsaturated carboxylic acids (α,β -UCAs)

Achieving the production of longer-chain ($C>4$) α,β -UCAs requires the incorporation of an enoyl-CoA reductase to enable a complete r-BOX and allow for its operation in a cyclical manner. For this purpose, trans-enoyl-CoA reductase from *E. glaucalis* (*egTER*) was integrated into the chromosome of JC01(DE3) along with other core r-BOX enzymes under the same promoter. When the resulting strain carrying vector pCDFDuet-p1-*ydiI* (JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}/pCDFDuet-p1-*ydiI*), was grown in shake flasks under the conditions described in previous sections, it produced 55 mg/L of medium-chain α,β -UCAs ($C_{6:1}$, $C_{8:1}$, $C_{10:1}$) compared to no synthesis of these products by JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5} (Figure 5A). In addition, crotonic acid production by JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}/pCDFDuet-p1-*ydiI* decreased to 0.9 g/L, compared to the 1.5 g/L produced by its parent strain JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5} (pCDFDuet-p1-*ydiI*) (Figure 5A). For the identification of these medium-chain α,β -UCAs, retention times of authentic standards were initially compared with the ones from extracted carboxylic acids (CAs). Subsequent fragmentation patterns analysis of extracted CAs with GC-MS library identified, as expected, both 2-hexenoic acid and 2-octenoic acid (Figure 6). 2-decenoic acid, however, was only identified by retention time analysis due to inconclusive mass spectrum data resulting from low titer and poor resolution.

Strain JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5} carrying pCDFDuet-p1-*ydiI* also produced significant amounts of acetate (3.5 g/L) and small amounts of butyrate (0.52 g/L), which indicates the presence of reactions that compete with thiolase BktB for acetyl-CoA and with thioesterase YdiI for enoyl-CoA intermediates. Given the low catalytic efficiency of

YdiI with respect to acetyl-CoA (Table 2), we assumed that other native thioesterases are responsible for the conversion of acetyl-CoA to acetate. In fact, overexpression of thioesterases YciA, YbgC, TesA, FadM, and TesB resulted in significant increases in acetate production (data not shown). We also hypothesized that some of the produced medium-chain α,β -UCAs might be re-utilized by the cells using a catabolic β -oxidation pathway, which can be avoided by the deletion of acyl-CoA dehydrogenase (*fadE*) (Clard and Cronan, 2005).

Based on aforementioned postulates, we created a thioesterase-deficient derivative of JC01(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}, which also contains a *fadE* deletion. Serial deletion of six thioesterases (*yciA*, *ybgC*, *ydiI*, *tesA*, *fadM*, *tesB*) along with *fadE* were carried out in JC01(DE3) resulting in *E. coli* strain JST07 (Kim et al., 2015), which was followed by incorporation of *bktB*, *fadB*, and *egTER* into the chromosome. The resulting strain, JST07(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}, carrying pCDFDuet-p1-*ydiI* produced 99 mg/L of medium-chain α,β -UCAs, a 2-fold improvement in the synthesis of these products (Figure 5A). We also assessed the impact of the expression level of core r-BOX enzymes on the production of medium-chain α,β -UCAs. To this end, we increased the concentration of inducer cumate and showed that synthesis of medium-chain α,β -UCAs by JST07(DE3)-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}/pCDFDuet-p1-*ydiI* can be increased by about 2-fold to reach 189 mg/L (Figure 5B). 2-octenoic acid (C_{8:1}) was the major product composing 65% of the mixture, followed by 30% 2-hexenoic acid (C_{6:1}) and 5% 2-decenoic acid (C_{10:1}) (Figure 5B).

3.5. Quantitative comparison of r-BOX and fatty acids biosynthesis pathways (FAB) as platforms for the production of α,β -unsaturated carboxylic acids

Since the results described in previous sections represent the first report of medium-chain α,β -UCAs production through the r-BOX pathway, we used a genome scale model and

constraint-based modeling to quantitatively establish the capabilities of this pathway for the synthesis of α,β -UCAs. Given that the fatty acid biosynthesis (FAB) pathway was recently exploited for the synthesis of crotonic acid (Liu et al., 2015), we compared r-BOX and FAB in the context of α,β -UCAs production. Simulations were conducted as described in *Materials and Methods*, under fermentative conditions, using an *E. coli* strain deficient in mixed acid fermentation pathways, and glucose as the carbon source.

When production of α,β -UCAs was simulated through the r-BOX pathway in a strain deficient in mixed-acid fermentation pathways, a coupling between cell growth and product synthesis was observed for all simulated chain lengths (Figure 7A). These results validate the feasibility of efficient production of α,β -UCAs through the r-BOX as the product synthesis pathway enables fermentative growth. However, no feasible solution was found when simulations were conducted under the same conditions, but using the FAB pathway instead of r-BOX. We hypothesize that the predicted inability of a strain deficient in mixed-acid fermentation pathways to synthesize α,β -UCAs through the FAB pathway is due to either a redox or an ATP imbalance under the simulated fermentative conditions.

The redox imbalance hypothesis was evaluated by restoring the ability to synthesize ethanol, which can provide a sink for reducing equivalents. Simulations under these conditions predicted a maximum rate of crotonic acid synthesis of 44 mmol C/g CDW/h for a non-growth scenario (i.e. zero specific growth rate) and no coupling between product synthesis and cell growth. Based on these results we concluded that a redox imbalance is not likely to prevent the growth-coupled synthesis of α,β -UCAs through the FAB pathway.

The hypothesis of ATP limitation, which arises from the fact that ATP is consumed in the FAB pathway to generate extender unit malonyl-ACP ($\text{acetyl-CoA} + \text{CO}_2 + \text{ATP} \rightarrow$

malonyl-CoA + ADP), was assessed by introducing an artificial ATP-independent acetyl-CoA carboxylase ($\text{acetyl-CoA} + \text{CO}_2 \rightarrow \text{malonyl-CoA}$) resulting in no ATP consumption in the generation of extender unit malonyl-ACP. Upon this modification, the model predicted a coupling between cell growth and synthesis of crotonic acid through the FAB pathway (Figure 7B). However, both cell growth and titer were significantly lower than observed when the r-BOX pathway was used (Table 3). This difference could be caused by the use of an NADPH-dependent 3-oxoacyl ACP reductase in the FAB pathway, while r-BOX uses an NADH-dependent 3-hydroxyacyl-CoA dehydrogenase. Supply of NADPH can be achieved through transhydrogenase PntAB, which is coupled to proton transport. This, in turn, can lead to more ATP consumption to maintain the electrochemical balance of protons and lower cell growth rate indicating that ATP is limiting the production of α,β -UCAs through the FAB pathway. The difference in cell growth rate between r-BOX and FAB on account of ATP balance results in differences in the biosynthesis of the target chemical since both product synthesis and cell growth are coupled. In this scenario, the model predicted that the titer of crotonic acid by r-BOX is 20% higher than that of FAB (Table 3).

Prior to our work no microorganism had been engineered to produce 2-hexenoic acid ($\text{C}_{6:1}$), 2-octenoic acid ($\text{C}_{8:1}$), or 2-decenoic acid ($\text{C}_{10:1}$), and hence the experimental performance of r-BOX and FAB pathways can only be compared for the production of crotonic acid (i.e. 2-butenic acid: $\text{C}_{4:1}$). For example, the FAB pathway was recently engineered in *E. coli* to produce crotonic acid, reaching a titer of 0.16 g/L with yield of 0.03 g/g glucose in shake flask fermentations (Liu et al., 2015). Since the experiments we report in previous sections were conducted using glycerol as the carbon source (as opposed to glucose in the study of Liu et al., 2015), we repeated them using glucose as the carbon source, which allows to establish a direct comparison between the two pathways. Using glucose as the

carbon source, our engineered strain produced 1.02 g/L of crotonic acid at a yield of 0.16 g/g glucose via r-BOX pathway (corresponding to 34% of the theoretical maximum 0.47g crotonate/g glucose), which is more than 5-fold higher than reported for the FAB pathway (Figure 7C).

Taken together, *in vivo* experiments coupled with *in silico* genome-wide stoichiometric analysis provides new evidence for the superior nature of the r-BOX in the production of α - β unsaturated carboxylic acids.

4. Conclusions

Successful conversion of glycerol, a renewable carbon source, to short- and medium-chain α , β -unsaturated carboxylic acids (α , β -UCAs) by an engineered *E. coli* strain was demonstrated. In addition to achieving 2-butenic acid (crotonic acid) production with a highly improved performance, our work represents the first report of 2-hexenoic acid, 2-octenoic acid, or 2-decenoic acid production via fermentation. Key aspects of our strategy include the engineering of a functional reversal of the β -oxidation pathway (r-BOX) capable of accumulating intracellular enoyl-CoAs and the expression of an enoyl-CoA-specific cycle-terminating thioesterase. The engineered functional r-BOX exhibited its best performance when a thiolase from *R. eutropha* (*bktB*) and native *E. coli* 3-hydroxyacyl-CoA dehydrogenase/enoyl-CoA hydratase (*fadB*) were chromosomally overexpressed under the control of a cumate inducible phage promoter. YdiI, a native *E. coli* thioesterase, was demonstrated to have the ability to convert a wide range of enoyl-CoAs to the corresponding α , β -UCAs, thus serving as the cycle-terminating enzyme. Kinetic analysis of YdiI showed that the activity on crotonyl-CoA is well described by Michaelis-Menten kinetics. This enzyme has very low catalytic efficiency toward acetyl-CoA, which makes it a promising

enzyme to achieve efficient product synthesis via the r-BOX platform. An *in silico* analysis revealed that the r-BOX is a superior platform for the synthesis of α,β -UCAs and identified the availability of ATP as a crucial factor causing a significant disparity in performance between r-BOX and the fatty acids biosynthesis pathway.

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Figure Captions

Figure 1. Engineered reversal of the β -oxidation cycle (r-BOX) for the synthesis of α,β -unsaturated carboxylic acids. Pyruvate formate-lyase is assumed to be the primary enzyme responsible for the dissimilation of pyruvate to acetyl-CoA (Durnin et al., 2009). Enzymes (*genes*) for each step used in this study are as follows (*E. coli* enzymes/*genes* unless otherwise stated): (1) thiolase (*atoB*, *fadA*, *Ralstonia eutropha bktB*); (2) 3-hydroxyacyl-CoA dehydrogenase (*fadB*); (3) enoyl-CoA hydratase (*fadB*); (4) enoyl-CoA reductase (*Euglena gracilis* trans-enoyl-CoA reductase: *egTER*); (5) thioesterase (*ydiI*).

Figure 2. Effect of thioesterase overexpression in the production of crotonic acid by strain MG1655(DE3)-*atoB*^{CT5}-*fadB*^{CT5}, which expresses AtoB and FadB from chromosomal constructs under the control of cumate-inducible promoter CT5. (A) Production of crotonic acid upon overexpression of different thioesterases in strain MG1655(DE3)-*atoB*^{CT5}-*fadB*^{CT5}. (B) GC-MS confirmation of crotonic acid biosynthesis by MG1655(DE3)-*atoB*^{CT5}-*fadB*^{CT5}/pCDFDuet-p1-*ydiI*, showing near perfect match of extracted sample to spectra for crotonic acid methyl ester. Each thioesterase was cloned into the pCDFDuet vector. 100 μ M of cumate was used for the expression of core r-BOX enzymes and 10 μ M of IPTG for thioesterase expression. The leading sequence of *tesA* was removed (*tesA'*).

Figure 3. Kinetic analysis of *E. coli* thioesterase YdiI on crotonyl-CoA. Data was fit to the Michaelis-Menten equation with $K_m = 524 \pm 18 \mu\text{M}$ and $k_{cat} = 0.93 \pm 0.03 \text{ s}^{-1}$.

Figure 4. Impact of gene deletion encoding fermentative enzymes and thiolase on (A) glycerol consumption, cell growth, and pH, (B) pyruvate, acetate, and butyrate production, and (C) crotonate production. Both JC01(DE3) (MG1655 $\Delta ldhA \Delta poxB \Delta pta \Delta adhE \Delta frdA$) and MG1655(DE3) have the same gene construction for the expression of key genes in r-BOX and *ydiI* (*atoB*^{CT5}-*fadB*^{CT5}/pCDFDuet-p1-*ydiI* or *bktB*^{CT5}-*fadB*^{CT5}/pCDFDuet-p1-*ydiI*). Bio-reactor operation was conducted using the same medium with controlled pH.

Figure 5. Production of medium chain α,β -unsaturated carboxylic acids ($C_{6:1}$, $C_{8:1}$, $C_{10:1}$). Core genes for r-BOX are under control of cumate inducible CT5 promoter while that of cycle terminating thioesterase (*ydiI*) is under a T7 promoter. (A) Impact of endogenous thioesterase deletion. The same set of genes for r-BOX and thioesterase (*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}/pCDFDuet-p1-*ydiI*) were overexpressed in both JC01(DE3) and JST07(DE3), (B) Impact of cumate concentration on production of medium chain α,β -unsaturated carboxylic acids in strain JST07-*bktB*^{CT5}-*fadB*^{CT5}-*egTER*^{CT5}/pCDFDuet-p1-*ydiI*. Concentration of IPTG was fixed at 10 μM .

Figure 6. Identification of 2-hexenoic acid (A) and 2-octenoic acid (B) by GC-MS. (A) and (B) Top: spectra of extracted carboxylic acid methyl ester. (A) and (B) Bottom: spectra for 2-hexenoic acid and 2-octenoic acid methyl esters obtained from the National Institute of Standards and Technology library.

Figure 7. *In silico* (A, B) and *in vivo* (C) analysis comparing α,β -unsaturated carboxylic acid (α,β -UCAs) production via engineered β -oxidation reversal (r-BOX) and fatty acid biosynthesis (FAB) pathways expressed in an *E. coli* strain deficient in mixed-acid fermentation pathways (i.e. equivalent to strain JC01 shown in Figure 3) and cultivated under fermentative conditions. (A), Solution space for the synthesis of α,β -UCAs through r-BOX. No production of α,β -UCAs was observed when the FAB pathway was used. (B), Synthesis of α,β -UCAs through FAB pathway using a hypothetical ATP-independent acetyl-CoA carboxylase ($\text{acetyl-CoA} + \text{CO}_2 \rightarrow \text{malonyl-CoA}$). (C), Crotonic acid production in flask fermentations of *E. coli* strains with engineered r-BOX (this study) and FAB pathway (Liu et al., 2015) using glucose as the carbon source.

Tables

Table 1. Strains and plasmids used in this study

Strains/Plasmids	Relevant Genotype/Property	Source
Strains		
MG1655	F- <i>l-ivG-rfb-50 rph-1</i>	Kang et al., 2004
HME45	W3110 $\Delta lacU169$ $\lambda c1857$ $\Delta(cro-bioA)$ HME45	Yu et al., 2000
JC01	MG1655, $\Delta ldhA::FRT$ $\Delta poxB::FRT$ $\Delta pta::FRT$ $\Delta adhE::FRT$ $\Delta frdA::FRT$ sequential deletion of <i>ldhA</i> , <i>poxB</i> , <i>pta</i> , <i>adhE</i> , and <i>frdA</i> harboring a λ DE3 Lysogen	Clomburg et al., 2012
JST07	JC01 $\Delta yciA$ $\Delta ybgC$ $\Delta ydiI$ $\Delta tesA$ $\Delta fadM$ $\Delta tesB$ $\Delta fadE$	Kim et al., 2015
MG1655 (DE3) <i>atoB</i> ^{CT5} <i>fadB</i> ^{CT5}	MG1655(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>atoB</i> FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$	This report
JC01 (DE3) <i>atoB</i> ^{CT5} <i>fadB</i> $\Delta fadA$	JC01(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>atoB</i> FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$	This report
JC01 (DE3) <i>bktB</i> ^{CT5} <i>fadB</i> $\Delta fadA$	JC01(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>btkB</i> $\Delta atoB$ FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$	This report
JC01 (DE3) <i>bktB</i> ^{CT5} <i>fadB</i> ^{CT5} $\Delta fadA$ <i>egTER</i> ^{CT5}	JC01(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>btkB</i> $\Delta atoB$ FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$ FRT- <i>cymR</i> -P ^{CT5} - <i>egTER</i>	Kim et al., 2015
JST07 (DE3) <i>bktB</i> ^{CT5} <i>fadB</i> ^{CT5}	JST07(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>btkB</i> $\Delta atoB$ FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$	This report
JST07 (DE3) <i>bktB</i> ^{CT5} <i>fadB</i> ^{CT5} $\Delta fadA$ <i>egTER</i> ^{CT5}	JST07(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>btkB</i> $\Delta atoB$ FRT- <i>cymR</i> -P ^{CT5} - <i>fadB</i> $\Delta fadA::zeo$ FRT- <i>cymR</i> -P ^{CT5} - <i>egTER</i>	Kim et al., 2015
JST07 (DE3) <i>bktB</i> ^{CT5} <i>fadBA</i> ^{CT5} <i>egTER</i> ^{CT5}	JST07(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>btkB</i> $\Delta atoB$ FRT- <i>cymR</i> -P ^{CT5} - <i>fadBA</i> FRT- <i>cymR</i> -P ^{CT5} - <i>egTER</i>	Kim et al., 2015
JST07 (DE3) <i>atoB</i> ^{CT5} <i>fadBA</i> ^{CT5} <i>egTER</i> ^{CT5}	JST07(DE3) FRT- <i>cymR</i> -P ^{CT5} - <i>atoB</i> FRT- <i>cymR</i> -P ^{CT5} - <i>fadBA</i> FRT- <i>cymR</i> -P ^{CT5} - <i>egTER</i>	Kim et al., 2015
Plasmids		
pCDFDuet	T ₇ promoter, CloDF13 origin, <i>lacI</i> , Sm ^R	EMD Novagen
pCDF- <i>fadM</i>	<i>E. coli</i> <i>fadM</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	Kim et al., 2015
pCDF- <i>tesA</i> '	<i>E. coli</i> <i>tesA</i> without leader sequence gene under <i>T7lac</i> promoter and <i>lacI</i> control	Kim et al., 2015
pCDF- <i>yciA</i>	<i>E. coli</i> <i>yciA</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This report
pCDF- <i>ybgC</i>	<i>E. coli</i> <i>ybgC</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This report

pCDF- <i>tesB</i>	<i>E. coli tesB</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This report
pCDF- <i>ydiI</i>	<i>E. coli ydiI</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This report
pET- <i>bsfabL</i>	<i>B. subtilis</i> (ATCC 23857) <i>fabL</i> gene under <i>T7-lac</i> and <i>lacI</i> control	Vick et al., 2015
pCDF- <i>ydiI</i> -his	<i>E. coli ydiI</i> N-terminal <i>his</i> tagged gene under <i>T7lac</i> promoter and <i>lacI</i> control	This report

Table 2. Comparison of kinetic parameters of YdiI with different substrates

Substrate	$k_{cat}(s^{-1})$	$K_m (\mu M)$	$k_{cat}/k_M (M^{-1}s^{-1})$	Reference
Acetyl-CoA	$(4.4 \pm 0.2) \times 10^{-3}$	$1,559 \pm 75$	2.8×10^{-6}	Chen et al., 2013
Crotonyl-CoA	$(9.3 \pm 0.03) \times 10^{-1}$	523 ± 18	1.8×10^3	This study
Hexanoyl-CoA	$(3.0 \pm 0.1) \times 10^{-1}$	21 ± 1	1.4×10^4	Latham et al., 2014
Lauroyl-CoA	$(7.4 \pm 0.1) \times 10^{-1}$	2.2 ± 0.1	3.3×10^5	Latham et al., 2014

Table 3. Comparison of specific growth rate, production rate, titer and yield for crotonic acid production obtained from *in silico* analysis for r-BOX and FAB pathways.

	FAB ¹	r-BOX
Specific growth rate (1/h)	0.04	0.08
Production rate (mol C/g DCW/h)	0.05	0.05
Titer (g/L) ²	15.5	19.8
Yield (mol C/mol C)	0.52	0.51

¹, A hypothetical ATP independent acetyl-CoA carboxylase was used.

², Initial cell concentration was set as 1 gCDW/L.

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Figure

Figure 1

A **B**

Figure 2

Figure 3

Figure 4

Figure 5

Figure 6

Figure 7