

# Synthesis of medium-chain length (C6–C10) fuels and chemicals via $\beta$ -oxidation reversal in *Escherichia coli*

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**Abstract** The recently engineered reversal of the  $\beta$ -oxidation cycle has been proposed as a potential platform for the efficient synthesis of longer chain ( $C \geq 4$ ) fuels and chemicals. Here, we demonstrate the utility of this platform for the synthesis of medium-chain length (C6–C10) products through the manipulation of key components of the pathway. Deletion of endogenous thioesterases provided a clean background in which the expression of various thiolase and termination components, along with required core enzymes, resulted in the ability to alter the chain length distribution and functionality of target products. This approach enabled the synthesis of medium-chain length carboxylic acids and primary alcohols from glycerol, a low-value feedstock. The use of BktB as the thiolase component with thioesterase TesA' as the termination enzyme enabled the synthesis of about 1.3 g/L C6–C10 saturated carboxylic acids. Tailoring of product formation to primary alcohol synthesis was achieved with the use of various acyl-CoA reductases. The combination of AtoB and FadA as the thiolase components with the alcohol-forming acyl-CoA reductase Maqu2507 from *M. aquaeolei* resulted in the synthesis of nearly 0.3 g/L C6–C10 alcohols. These results further demonstrate the versatile nature of a  $\beta$ -oxidation reversal, and highlight

several key aspects and control points that can be further manipulated to fine-tune the synthesis of various fuels and chemicals.

**Keywords**  $\beta$ -Oxidation reversal · Metabolic engineering · Synthetic biology · Fuels and chemicals · Medium-chain acids · Medium-chain alcohols

## Introduction

Modern society relies on fossil fuels as the main feedstock for the production of transportation fuels and commodity and fine chemicals. Due to the concerns over sustainability, increasing demand, and localized distribution of fossil feedstocks, price increases and instability are a common concern. These factors, along with environmental concerns, have stimulated the development of alternative methods for fuel and chemical production [32]. Biomass is regarded as an attractive alternative resource due to its sustainability, and, in comparison to fossil fuels, lower environmental impact and more uniform distribution throughout the world. However, the use of biomass as an alternative to fossil fuels requires the development of processes able to efficiently convert biomass feedstocks into a variety of important fuels and chemicals.

While the biological production of short chain products, such as ethanol, from biomass constituents has been demonstrated on the commercial scale, the production of medium to long-chain products ( $C \geq 6$ ) is far less established [1, 14]. This is in large part due to the fact that the production of these compounds must take place from 1-, 2- or 3-carbon metabolic intermediates that result from substrate utilization pathways and central carbon metabolism, and as such, require efficient carbon–carbon elongation

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pathways for their synthesis. These elongation pathways typically require additional energy and cofactors, which in turn result in lower yields and productivity compared to shorter chain molecules. Thus, the exploitation of elongation pathways that are carbon and energy efficient is highly desirable to maximize product yields.

The recently engineered reversal of the  $\beta$ -oxidation cycle provides one such pathway that has been proposed as a potential platform to enable the synthesis of short-, medium-, and long-chain products at high yields [5, 6, 10, 18, 37]. This pathway operates with coenzyme A (CoA) intermediates and directly uses acetyl-CoA for acyl-CoA elongation, characteristics that support product synthesis and maximum carbon and energy efficiency. In addition, the modular and versatile nature of this pathway provides the ability to generate a diverse set of products through the manipulation of any of the modules comprising a  $\beta$ -oxidation reversal [3].

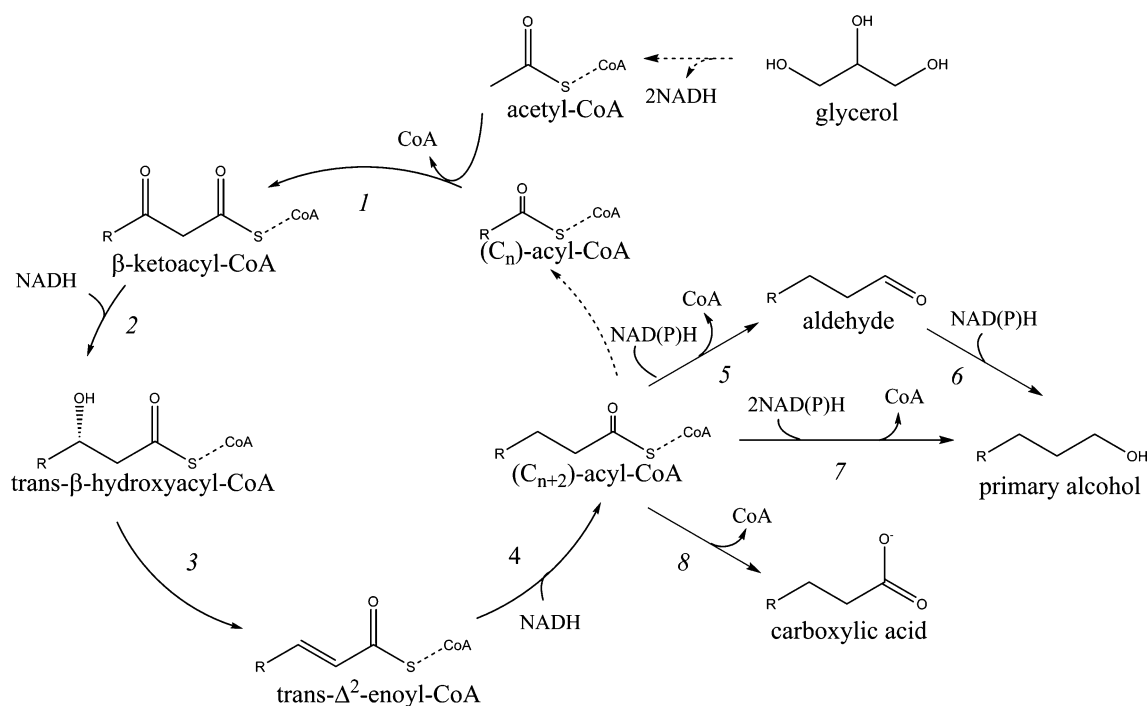
In this study, we sought to exploit a  $\beta$ -oxidation reversal to target the synthesis of medium-chain length (C6–C10) products of varying functionality. Key to this approach is increasing the generation and availability of target chain length acyl-CoA intermediates that can be converted to desired products through appropriate termination pathways (Fig. 1). This was achieved through the use of various thiolase enzyme(s) involved in the condensation reaction of

an acyl-CoA intermediate with acetyl-CoA (Fig. 1), as well as the manipulation of the termination enzyme(s) utilized, representing the synthesis of specific products from intermediates generated through a  $\beta$ -oxidation reversal (Fig. 1). The latter included not only the expression of candidate enzymes for carboxylic acid and alcohol production, but also the deletion of native thioesterases that can influence the availability of a given chain length acyl-CoA. This approach provides the opportunity to control both the chain length and functionality of products synthesized through a reversal of the  $\beta$ -oxidation cycle and demonstrate how this pathway can be exploited for the synthesis of a wide array of compounds.

## Materials and methods

### Strains, plasmids, and genetic methods

Wild-type K12 *Escherichia coli* strain MG1655 [15] 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 [23]. JST07 (DE3) was constructed from JST07 using a  $\lambda$ DE3 Lysogenization Kit (Novagen,



**Fig. 1** Engineered  $\beta$ -oxidation reversal for the synthesis of saturated carboxylic acids and primary alcohols. Enzymes for each step investigated in this study are as follows (gene names in parentheses): 1 thiolase (*atoB*, *bktB*, *fadA*, *yqeF*); 2 hydroxyacyl-CoA dehydrogenase (*fadB*); 3 enoyl-CoA hydratase (*fadB*); 4 enoyl-CoA reduc-

tase (*egTER*); 5 aldehyde-forming acyl-CoA reductase (*ald*, *acr1*, *acrM*, *eutE*, *mhpF*, *orf1594*); 6 alcohol/aldehyde dehydrogenase (*adhP*, *fucO*, *yqhD*); 7 alcohol-forming acyl-CoA reductase (*adhE2*, *maqu2220*, *maqu2507*); 8 thioesterase (*tesA*, *tesB*, *fadM*, *ybcC*, *yciA*, *ydiI*)

**Table 1** Strains and plasmids used in this study

Strains/plasmids	Relevant genotype/description	Source
<b>Strains<sup>a</sup></b>		
MG1655	F- <i>l-ivlG-rfb-50 rph-1</i>	[15]
JC01	MG1655, $\Delta$ <i>ldhA</i> ::FRT $\Delta$ <i>poxB</i> ::FRT $\Delta$ <i>pta</i> ::FRT $\Delta$ <i>adhE</i> ::FRT $\Delta$ <i>frdA</i> ::FRT	[5]
JC01 (DE3)	JC01 harboring a $\lambda$ DE3 Lysogen	[33]
JC01 (DE3) $\Delta$ <i>fadE</i>	JC01 (DE3) $\Delta$ <i>fadE</i> ::FRT	This study
JST01	JC01 $\Delta$ <i>yciA</i> ::FRT	This study
JST02	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT	This study
JST03	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT $\Delta$ <i>ydil</i> ::FRT	This study
JST04	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT $\Delta$ <i>ydil</i> ::FRT $\Delta$ <i>tesA</i> ::FRT	This study
JST05	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT $\Delta$ <i>ydil</i> ::FRT $\Delta$ <i>tesA</i> ::FRT $\Delta$ <i>fadM</i> ::FRT	This study
JST06	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT $\Delta$ <i>ydil</i> ::FRT $\Delta$ <i>tesA</i> ::FRT $\Delta$ <i>fadM</i> ::FRT $\Delta$ <i>tesB</i> ::FRT	This study
JST07	JC01 $\Delta$ <i>yciA</i> ::FRT $\Delta$ <i>ybgC</i> ::FRT $\Delta$ <i>ydil</i> ::FRT $\Delta$ <i>tesA</i> ::FRT $\Delta$ <i>fadM</i> ::FRT $\Delta$ <i>tesB</i> ::FRT $\Delta$ <i>fadE</i> ::FRT	This study
JST07 (DE3)	JST07 harboring a $\lambda$ DE3 Lysogen	This study
JC01 (DE3) <i>bktB</i> <sup>CT5</sup> <i>fadB</i> <sup>CT5</sup> $\Delta$ <i>fadA</i> <i>egTER</i> <sup>CT5</sup>	JC01 (DE3) FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>fadB</i> $\Delta$ <i>fadA</i> ::zeo FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>bktB</i> $\Delta$ <i>atoB</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>egTER</i> @ <i>fabI</i> chromosomal location	[4]
JST07 (DE3) <i>atoB</i> <sup>CT5</sup> <i>fadB</i> <sup>CT5</sup> $\Delta$ <i>fadA</i> <i>egTER</i> <sup>CT5</sup>	JST07 (DE3) FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>fadB</i> $\Delta$ <i>fadA</i> ::zeo FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>atoB</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>egTER</i> @ <i>fabI</i> chromosomal location	This study
JST07 (DE3) <i>fadBA</i> <sup>CT5</sup> <i>egTER</i> <sup>CT5</sup>	JST07 (DE3) FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>fadBA</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>egTER</i> @ <i>fabI</i> chromosomal location	This study
JST07 (DE3) <i>atoB</i> <sup>CT5</sup> <i>fadBA</i> <sup>CT5</sup> <i>egTER</i> <sup>CT5</sup>	JST07 (DE3) FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>fadBA</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>atoB</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>egTER</i> @ <i>fabI</i> chromosomal location	This study
JST07 (DE3) <i>bktB</i> <sup>CT5</sup> <i>fadB</i> <sup>CT5</sup> $\Delta$ <i>fadA</i> <i>egTER</i> <sup>CT5</sup>	JST07 (DE3) FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>fadB</i> $\Delta$ <i>fadA</i> ::zeo FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>bktB</i> $\Delta$ <i>atoB</i> FRT- <i>cymR</i> -P <sup>CT5</sup> - <i>egTER</i> @ <i>fabI</i> chromosomal location	This study
<b>Plasmids</b>		
pTrcHis2A	P <sub>trc</sub> promoter, pBR322 origin, <i>lacI</i> , Amp <sup>R</sup>	Invitrogen
pCDFDuet	T <sub>7</sub> promoter, CloDF13 origin, <i>lacI</i> , Sm <sup>R</sup>	Novagen
pTH- <i>atoB</i> - <i>fadB</i> - <i>egTER</i>	<i>E. coli</i> <i>atoB</i> , <i>fadB</i> and <i>Euglena glaucilis</i> <i>TER</i> genes under <i>T7lac</i> promoter and <i>lacI</i> control	[5]
pCDF-P1- <i>fadM</i>	<i>E. coli</i> <i>fadM</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>tesA</i>	<i>E. coli</i> <i>tesA</i> without leader sequence gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>acr1</i>	<i>Acinetobacter calcoaceticus</i> <i>acr1</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>acrM</i>	<i>Acinetobacter</i> sp. <i>acrM</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>adhE2</i>	<i>Clostridium acetobutylicum</i> <i>adhE2</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>ald</i>	<i>Clostridium beijerinckii</i> <i>ald</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>eutE</i>	<i>Salmonella typhimurium</i> <i>eutE</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>mhpF</i>	<i>E. coli</i> <i>mhpF</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>maqu2220</i>	<i>Marinobacter aquaeolei</i> <i>maqu2220</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>maqu2507</i>	<i>Marinobacter aquaeolei</i> <i>maqu2507</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>orf1594</i>	<i>Synechococcus elongatus</i> <i>orf1594</i> gene under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>ald</i> -P2- <i>adhP</i>	<i>Clostridium beijerinckii</i> <i>ald</i> and <i>E. coli</i> <i>adhP</i> genes under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>ald</i> -P2- <i>fucO</i>	<i>Clostridium beijerinckii</i> <i>ald</i> and <i>E. coli</i> <i>fucO</i> genes under <i>T7lac</i> promoter and <i>lacI</i> control	This study
pCDF-P1- <i>ald</i> -P2- <i>yqhD</i>	<i>Clostridium beijerinckii</i> <i>ald</i> and <i>E. coli</i> <i>yqhD</i> genes under <i>T7lac</i> promoter and <i>lacI</i> control	This study

<sup>a</sup> CT5 designates cumate-controlled chromosomal expression construct of indicated gene(s) (see text for details)

Darmstadt, Germany) to allow the expression of genes under the T7 *lac* promoter. All resulting strains used in this study are listed in Table 1. Creation of constructs for the cumate-controlled chromosomal expression of *atoB* [34], *fadB*  $\Delta$ *fadA* [34], *fadBA* [34], *bktB*  $\Delta$ *atoB* [4], and *egTER* at the *fabI* chromosomal locus [4] were

achieved via standard recombineering protocols as previously described. For the chromosomal expression of *yqeF* under cumate control, *yqeF* was PCR amplified from *E. coli* MG1655 genomic DNA with primers containing homology for recombination in an HME45 strain containing an *atoB*<sup>CT5</sup>-*cat*-*sacB* insertion cassette [4].

This PCR product was then recombineered into the above HME45 strain, resulting in a *kan-cymR-P<sup>CT5</sup>-yqeF ΔatoB* construct at the *atoB* locus after negative selection on sucrose plates.

Plasmid-based gene overexpression was achieved by cloning the desired gene(s) into pCDFDuet-1 (Novagen, Darmstadt, Germany) digested with appropriate restriction enzymes utilizing In-Fusion PCR cloning technology (Clontech Laboratories, Inc., Mountain View, CA). Cloning inserts were created via PCR of ORFs of interest from their respective codon-optimized (*acr1*, *acrM*, *maqu2220*, *maqu2507*, *orf1594*) or genomic DNA (all others genes) with Phusion polymerase (Thermo Scientific, Waltham, MA, USA). The resulting In-Fusion products were used to transform *E. coli* Stellar cells (Clontech Laboratories, Inc., Mountain View, CA, USA) and PCR identified clones were confirmed by DNA sequencing.

All molecular biology techniques were performed with standard methods [23, 30] or following manufacturer protocol. Strains were kept in glycerol stocks at  $-80\text{ }^{\circ}\text{C}$ . Plates were prepared using LB medium containing 1.5 % agar, and appropriate antibiotics were included at the following concentrations: ampicillin (100  $\mu\text{g}/\text{mL}$ ), kanamycin (50  $\mu\text{g}/\text{mL}$ ), spectinomycin (50  $\mu\text{g}/\text{mL}$ ) and chloramphenicol (12.5  $\mu\text{g}/\text{mL}$ ).

#### Culture medium and cultivation conditions

The minimal medium designed by Neidhardt et al. [24], with 125 mM MOPS and  $\text{Na}_2\text{HPO}_4$  in place of  $\text{K}_2\text{HPO}_4$ , supplemented with 20 g/L glycerol, 10 g/L tryptone, 5 g/L yeast extract, 5 mM calcium pantothenate, 2.78 mM  $\text{Na}_2\text{HPO}_4$ , 5 mM  $(\text{NH}_4)_2\text{SO}_4$ , 30 mM  $\text{NH}_4\text{Cl}$ , 5  $\mu\text{M}$  sodium selenite, 100  $\mu\text{M}$  biotin, and 1 mg/L thiamine-HCl was used for all fermentations. Antibiotics (100 mg/L of ampicillin for pTH containing strains or 50  $\mu\text{g}/\text{mL}$  spectinomycin for pCDF containing strains) and inducers (isopropyl  $\beta$ -D-1-thiogalactopyranoside and cumate) were included when appropriate. All chemicals were obtained from Fisher Scientific Co. (Pittsburg, PA, USA) and Sigma-Aldrich Co. (St. Louis, MO, USA).

Fermentations were conducted under microaerobic conditions in 25-mL Pyrex Erlenmeyer flasks (narrow mouth/heavy duty rim, Corning Inc., Corning, NY, USA) filled with 15 mL of the above culture medium and sealed with foam plugs filling the necks [22]. A single colony of the desired strain was cultivated overnight (14–16 h) in LB medium with appropriate antibiotics and used as the inoculum (1 %) for all fermentations. After inoculation, flasks were incubated at  $37\text{ }^{\circ}\text{C}$  and 200 rpm in an NBS C24 Benchtop Incubator Shaker (New Brunswick Scientific Co., Inc., Edison, NJ, USA) until an optical density of  $\sim 0.3$ – $0.5$  was reached, at which point IPTG (10  $\mu\text{M}$ ) and/or cumate

(0.1 mM) were added when appropriate. Flasks were then incubated under the same conditions for 48 h post-induction unless otherwise stated.

#### Analytical methods

Optical density was measured at 550 nm in a Thermo Spectronic Genesys 20 (Thermo Scientific, Waltham, MA, USA) and used as an estimate of cell mass (1 OD 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, USA) equipped with an HPX-87H organic acid column (Bio-Rad, Hercules, CA, USA) with operating conditions to optimize peak separation (0.3 mL/min flowrate, 30 mM  $\text{H}_2\text{SO}_4$  mobile phase, column temperature  $42\text{ }^{\circ}\text{C}$ ).

Quantification of carboxylic acids ( $\text{C} > 6$ ) was conducted via GC-FID analysis using a Varian CP-3800 (Varian Associates, Inc., Palo Alto, CA, USA) equipped with a flame ionization detector (GC-FID) and an HP-5 capillary column (0.25 mm internal diameter, 0.25  $\mu\text{m}$  film thickness, 30 m length; Agilent Technologies, Santa Clara, CA, USA). Sample preparation was conducted as follows: 2 mL culture supernatant samples were transferred to 5-mL glass vials (Fisher Scientific Co., Fair Lawn, NJ, USA) and 80  $\mu\text{L}$  of 50 %  $\text{H}_2\text{SO}_4$  and 340  $\mu\text{L}$  of 30 % NaCl solution were added for pH and ionic strength adjustment, respectively. Tridecanoic acid (final concentration 50 mg/L) was added as internal standard and 2 mL of hexane-MTBE (1:1) added for extraction. The bottles were sealed with Teflon-lined septa (Fisher Scientific Co., Fair Lawn, NJ, USA), secured with caps, and rotated at 60 rpm for 120 min. The samples were then centrifuged for 2 min at  $2,375\times g$  to separate the aqueous and organic layers. After centrifugation, 1.5 mL of the top organic layer was separated from the aqueous layer. Each extracted sample was transferred to 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 carboxylic acids to fatty acid methyl esters (FAMES), and the vials capped tightly and vortexed. These vials were then placed in heater block (AccuBlock digital dry bath; LabNew, Woodbridge, NJ, USA) and heated at  $60\text{ }^{\circ}\text{C}$  for 60 min. The transesterification reaction vials were cooled to room temperature, followed by the addition of 1 mL of water and vortexing. FAMES were extracted with 1.5 mL hexane for 60 min at 60 rpm. The vials were then vortexed for 30 s and centrifuged for 2 min at  $2,375\times g$  to separate the aqueous and

organic layers. The upper organic phase collected with a glass syringe and transferred to GC vials (Fisher Scientific Co., Fair Lawn, NJ, USA) for GC analysis according to the following method: 1  $\mu$ L were injected into the GC, which was run in splitless mode using helium gas as a carrier gas with a flow rate of 1 mL/min. The injector temperature was 280 °C and the oven temperature was initially held at 50 °C for 3 min and then raised to 250 °C at 10 °C/min and held for 3 min. Analysis of primary alcohols followed the same procedure except 50  $\mu$ L of 2 mg/mL tridecanol in ethanol was added to the initial sample as an internal standard (in addition to the carboxylic acid internal standard) and a 1:1 pyridine–acetic anhydride mixture was used in place of the boron trifluoride-methanol solution for the acetylation of extracted primary alcohols followed by heating at 80 °C for 120 min.

### Enzyme assays

Cells were grown overnight in 25 mL LB medium in 125-mL Erlenmeyer flasks at 37 °C and 200 rpm, harvested, washed twice with a 9 g/L NaCl solution, and stored as cell pellets at –80 °C. Cell disruption was carried out by using glass (0.1 mm diameter) beads. The measurement of thioesterase (TE) activity was carried out by monitoring reduction of 5,5'-dithiobis(2-nitrobenzoic acid) (DTNB) [38]. The reaction mixture contained 0.1 M Tris–HCl (pH 7.5), 0.2 M KCl, 1 mM DTNB, 25  $\mu$ M acyl-CoA and crude extract in a final volume of 200  $\mu$ L. Reactions were carried out at 30 °C and DTNB reduction was measured by increase in optical density at 412 nm on a Synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT, USA). For the specific activity assays (reported in nmol substrate/mg protein/min), protein concentration was established using the Bradford Reagent (Thermo Scientific, Waltham, MA, USA) using BSA as the protein standard.

## Results and discussion

Reduction of endogenous thioesterase activity provides an efficient background to fine-tune the operation of a  $\beta$ -oxidation reversal

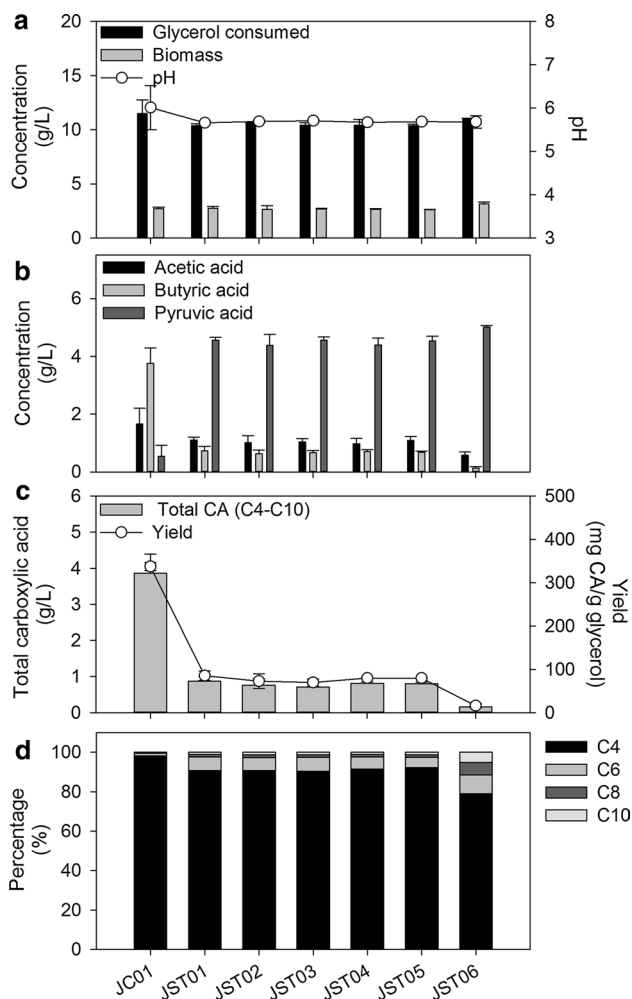
Previously, we have utilized a synthetic approach to construct and functionally characterize a reversal of the  $\beta$ -oxidation cycle [5] establishing the key core/elongation modules (thiolase, 3-hydroxyacyl-CoA dehydrogenase, 3-hydroxyacyl-CoA dehydratase, and trans-enoyl-CoA reductase) required for longer chain ( $C \geq 4$ ) product synthesis (Fig. 1). Using this approach, a one-turn reversal was constructed through the use of AtoB (thiolase), FadB (3-hydroxyacyl-CoA dehydrogenase and

3-hydroxyacyl-CoA dehydratase), and the trans-2-enoyl reductase from *E. gracilis* (egTER) in an *E. coli* strain devoid of native fermentation pathways (JC01: MG1655  $\Delta$ ldhA  $\Delta$ pta  $\Delta$ poxB  $\Delta$ adhE  $\Delta$ frdA). A multiple turn  $\beta$ -oxidation reversal was facilitated through the use of the longer chain length specific thiolase FadA in addition to AtoB, FadB, and egTER [5]. Product synthesis was demonstrated in the form of carboxylic acids resulting from the expression of these core components without the controlled overexpression of a termination pathway. While this established the key core/elongation components required, the fact that endogenous enzymes resulted in carboxylic acid production represents a significant issue with regard to fine-tuning both the chain length and type(s) of products synthesized.

In order to provide a background more amenable to fine-tuning product synthesis, we sought to eliminate as many endogenous termination pathways as possible. This will not only enable the selection of specific termination pathways for the chain length and type of product desired, but can also facilitate increases in the intracellular pool(s) of required acyl-CoA intermediates by minimizing their conversion to undesired products. Considering the carboxylic acid production observed with only the expression of the core/elongation enzymes, native thioesterases capable of hydrolyzing acyl-CoA intermediates are candidates for the endogenous enzymes leading to product synthesis. *E. coli* possesses several enzymes that could be functioning in this capacity, including the thioesterases encoded by *tesA* [2], *tesB* [25], *yciA* [38], *fadM* [7], *ydiI* [16], and *ybgC* [11]. Serial deletion of these six genes in JC01 (MG1655  $\Delta$ ldhA  $\Delta$ pta  $\Delta$ poxB  $\Delta$ adhE  $\Delta$ frdA) was undertaken. Using butyrate production as a proxy for product synthesis from a one-turn  $\beta$ -oxidation reversal with AtoB, FadB, and egTER expression, the deletion of these genes had a dramatic impact on product synthesis as the production of acetate and butyrate decreased significantly with a corresponding increase in pyruvate generation (Fig. 2). While the order of deletion could play a role in the influence of a specific thioesterase deletion, it should be noted that *yciA* and *tesB* deletions appear to have the most significant impact on the titers of carboxylic acids under these conditions (Fig. 2). The construction of this “thioesterases-deficient” strain should provide a more efficient background for operation of multiple cycle turns, which in turn can facilitate the synthesis of target products upon the controlled expression of termination pathways of interest.

While the above results demonstrated the reduction in shorter chain carboxylic acid synthesis, in the context fine-tuning medium-chain length product synthesis from a  $\beta$ -oxidation reversal, it is also important to determine the impact these thioesterase deletions have on the cleavage of longer chain length acyl-CoA intermediates. This was





**Fig. 2** Impact of endogenous thioesterase deletion on (a) cell growth, pH, and glycerol consumption, (b) acetic, butyric, and pyruvic acids, (c) total C4–C10 saturated carboxylic acid titer, and (d) C4–C10 saturated carboxylic acid distribution. Components for a functional  $\beta$ -oxidation reversal (AtoB, FadB, and *egTER*) were expressed from pTrcHis2A in all strains. Serial deletion of thioesterases in JC01 (MG1655  $\Delta dhA \Delta pta \Delta poxB \Delta adhE \Delta fzdA$ ) in the following order with JST06 representing JC01 will all 6 deletions: *yciA*, *ybgC*, *ydlI*, *tesA*, *fadM*, *tesB* (see text for details)

evaluated through the measurement of acyl-CoA hydrolysis activities from crude cell extracts of strains JC01 (DE3)  $\Delta fadE$  and JST07 (DE3) with the serial deletion of the six thioesterases described above being the difference between the two strains (Table 2). It should be noted that each of these strains contains a deletion of *fadE*, encoding the acyl-CoA dehydrogenase responsible for the degradation of acyl-CoA intermediates in the catabolic operation of the  $\beta$ -oxidation cycle, as well as a  $\lambda$ DE3 Lysogen to allow the expression of genes under the T7 *lac* promoter. As seen in Table 2, JST07 (DE3) exhibits significantly lower activities for the hydrolysis of all C2–C10 saturated acyl-CoAs tested, when compared to JC01 (DE3)  $\Delta fadE$ . Based on

**Table 2** Specific thioesterase activity (nmol/mg protein/min) of background strains on various chain length acyl-CoA intermediates

Substrate	Strain	
	JC01 (DE3) $\Delta fadE$	JST07 (DE3)
Acetyl-CoA	17.2 ± 10.8	2.3 ± 2.2
Butyryl-CoA	16.8 ± 0.7	2.0 ± 1.9
Hexanoyl-CoA	33.8 ± 1.2	0.0
Octanoyl-CoA	40.8 ± 0.9	2.5 ± 1.0
Decanoyl-CoA	26.6 ± 5.0	5.2 ± 3.2

these findings, JST07 (DE3) provides the ideal background for a multiple cycle turn reversal, as the minimization of acyl-CoA cleavage should enable the generation of significant levels of medium-chain acyl-CoA intermediates, which can be converted to products of interest through the expression of termination pathways exhibiting appropriate chain length specificity.

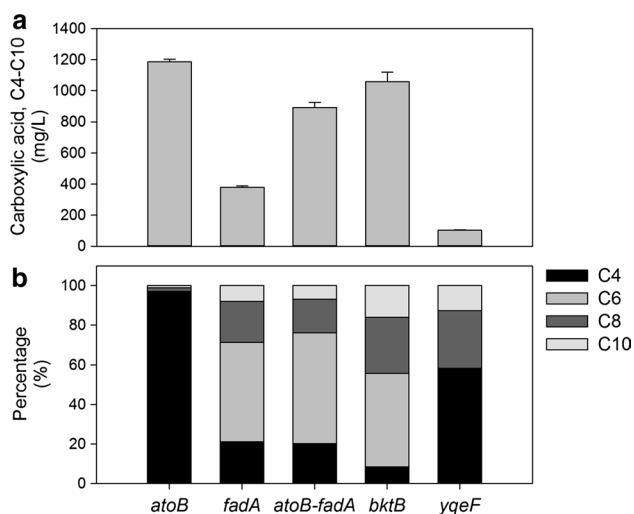
Controlling product chain length through the selection of appropriate thiolase and termination enzymes

While the deletion of endogenous thioesterases lowered acyl-CoA hydrolysis activities and minimized saturated carboxylic acid production with the expression of enzymes required for a one-turn  $\beta$ -oxidation reversal, the synthesis of medium-chain length products requires both the operation of multiple cycle turns as well as the expression of termination pathways with target chain length specificity. Given the broad chain length specificity of FadB and *egTER*, the ability to operate multiple cycles depends on the selection of thiolase(s) with the ability to efficiently condense acetyl-CoA with the growing chain length acyl-CoA intermediates (Fig. 1). For the synthesis of medium-chain length (C6–C10) products, thiolases that facilitate the generation of C6–C10 acyl-CoAs are required to provide an adequate intracellular pool of these key intermediates. Potential thiolases for use in this context include the aforementioned AtoB and FadA, along with the predicted *E. coli* acetyl-CoA acetyltransferase YqeF [28] and the  $\beta$ -ketothiolase BktB from *Ralstonia eutropha*, which has been shown to condense acetyl-CoA with C4 and C6 acyl-CoA intermediates [20, 21].

We chose to employ expression mechanisms allowing for the independent control of core/elongation and termination enzymes. For this, chromosomal expression of core/elongation enzymes under a cumate-inducible promoter was selected based on our previous results with the expression of AtoB, BktB, FadB(A), and/or *egTER* [4, 34]. For YqeF expression, a cumate-controlled expression construct was created in which *yqeF* seamlessly replaced *atoB* for expression at the *atoB* locus. Individual constructs for each

of these various thiolases were then combined with FadB and *egTER* constructs in JST07 (DE3) to enable full core/elongation module expression in this background strain with minimized endogenous thioesterase expression. While these strains have all required core/elongation enzymes for a functional  $\beta$ -oxidation reversal, the lack of acyl-CoA hydrolysis activity in this background (Table 2) requires the controlled expression of a thioesterase to enable product synthesis at high levels. For this purpose, *tesA'* (leaderless version of *tesA* [2]) was selected. *TesA'* was selected due in part to our preliminary characterization in which crude extracts of cells expressing *tesA* showed  $\sim$ tenfold higher specific activities on decanoyl-CoA compared to butyryl-CoA and other short chain CoA intermediates [5]. Considering the competition between the condensation and termination enzymes for the growing chain length acyl-CoA intermediates, these properties should minimize the cleavage of shorter-chain acyl-CoAs, while functioning for the hydrolysis reaction after medium-chain length acyl-CoAs are generated through a multiple turn  $\beta$ -oxidation reversal.

*tesA'* was cloned into a pCDF vector to enable expression independently from the core/elongation enzymes. As seen in Fig. 3, with *tesA'* expression, the production of various amounts of C4–C10 saturated carboxylic acids were observed dependent on the choice of thiolase in combination with FadB and *egTER*. The use of AtoB as the only thiolase component results in butyric acid as the only C4 or longer saturated carboxylic acid product (Fig. 3). However, the use of a longer chain length specific thiolase, such as FadA or BktB, enables the synthesis of longer chain



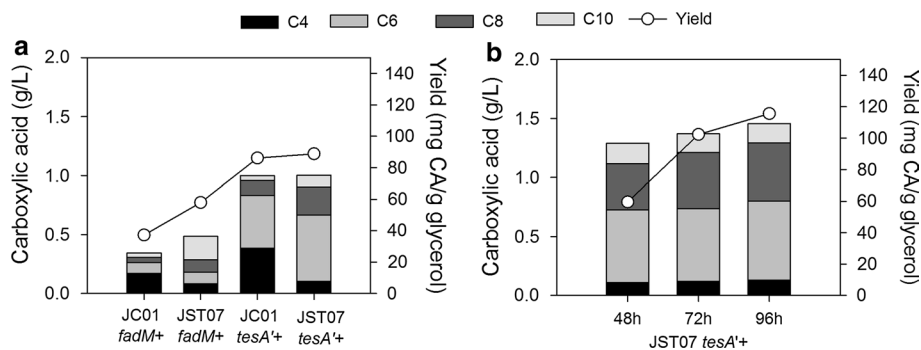
**Fig. 3** Influence of thiolase components on carboxylic acid production from a  $\beta$ -oxidation reversal. C4–C10 saturated carboxylic acid (a) titer and (b) distribution from JST07 (DE3) containing chromosomal constructs for the expression of *fadB*, *egTER*, and the indicated thiolase(s). *TesA'* (expressed from pCDF-P1-*tesA'*) was used for thioesterase termination

length carboxylic acids. Given the longer chain length specificity of FadA, however, its expression needs to be combined with that of AtoB in order to increase the synthesis of C4–C10 carboxylic acids, as more than a twofold increase in total titer was observed with AtoB and FadA expression compared to FadA as the sole thiolase (Fig. 3). The use of BktB as the sole thiolase component yielded an improved performance on both titer and selectivity towards medium-chain length carboxylic acids, as the synthesis of greater than 1 g/L total C4–C10 saturated carboxylic acids were observed with about 90 % of the products being C6–C10 (Fig. 3). These characteristics make this strain ideally suited to targeting medium-chain length (C6–C10) products.

While the above results demonstrate the influence of the thiolase component on product chain length from a  $\beta$ -oxidation reversal, further refinement to this distribution can potentially be achieved through the use of thioesterases with varying chain length specificity. Given the lower titers of decanoic acid in comparison to hexanoic and octanoic acids observed with *tesA'* expression with BktB as the thiolase (Fig. 3), we looked to employ a longer chain length specific thioesterase in an attempt to increase C10 production. For this purpose, the long-chain acyl-CoA thioesterase encoded by *fadM* from *E. coli* was cloned into pCDF for expression. Upon overexpression of *fadM* in JST07 (DE3) with the BktB, FadB, and *egTER* chromosomal expression constructs, a significant increase in decanoic acid production was observed compared to that with *tesA'* expression (Fig. 4A). This also corresponded to lower levels of C6 and C8 saturated carboxylic acid production, demonstrating the ability of the termination pathway to influence the chain length distribution (Fig. 4a). This type of fine-tuning of product synthesis was only achieved with *fadM* overexpression in JST07 (DE3), as significantly lower levels of decanoic acid were produced in JC01 (DE3) with the same core/elongation components and *fadM* expression (Fig. 4a).

The same trend described above for *fadM* overexpression was also observed with *tesA'* expression in the two backgrounds, as the use of *tesA'* in JST07 (DE3) resulted in higher C6, C8, and C10 titers with decreased butyrate titer in comparison to JC01 (DE3) (Fig. 4a). Further increase in medium-chain (C6–C10) carboxylic acid production was observed when strain JST07 (DE3) with *tesA'* overexpression was cultivated for an extended fermentation time and using higher concentration of inducer (200  $\mu$ M cumate) (Fig. 4b).

Overall, these results demonstrate the importance of manipulating both the thiolase component and termination pathways to ensure the generation of medium-chain length acyl-CoA intermediates available for product synthesis. The deletion of endogenous thioesterases with the use of appropriate thiolase(s) provides a background in which sufficient



**Fig. 4** Controlling carboxylic acid chain length through the manipulation of termination pathways (a) and improving titer of carboxylic acid (b). a C4–C10 saturated carboxylic acid titer and yield in various backgrounds with thioesterases *TesA'* or *FadM* as termination enzymes. Both JC01 (DE3) and JST07 (DE3) contain constructs for *bktB*, *fadB*, and *egTER* chromosomal expression with *tesA'* or

*fadM* expressed from plasmid pCDF. 10  $\mu$ M IPTG and 100  $\mu$ M cumate were used for expression; b Carboxylic acid production in JST07(DE3) with *bktB*, *fadB*, and *egTER* chromosomal expression and *tesA'* overexpression from plasmid pCDF at extended fermentation time. 10  $\mu$ M IPTG and 200  $\mu$ M of cumate were used for expression

supply of medium-chain length acyl-CoAs are generated. The overexpression of a thioesterase with desirable properties (i.e., sufficient activity on medium-chain acyl-CoAs with low activity for short chain acyl-CoA intermediates) can then be utilized to promote the synthesis of desired chain length carboxylic acids while minimizing the loss of carbon from the cleavage of shorter chain length CoA intermediates. While a more rigorous screening of potential acyl-CoA thioesterases is envisioned as a means to increase the total titer and chain length specificity of carboxylic acid synthesis, the ~1.3 g/L of medium-chain (C6–C10) carboxylic acids represent the largest concentration of these products reported in the literature. Moreover, the improved strain background in regard to the generation of medium-chain length acyl-CoAs enables the use of additional termination pathways for varying product functionality.

#### Synthesis of medium-chain length primary alcohols through a $\beta$ -oxidation reversal

With the efficiency of the  $\beta$ -oxidation reversal for medium-chain acyl-CoA generation improved through the deletion of native thioesterases and use of proper thiolases, the next area of investigation involved the overexpression of termination enzymes for the production of medium-chain length primary alcohols. This requires the use of various alcohol/aldehyde-forming acyl-CoA reductases or CoA-dependent dehydrogenases that can reduce target acyl-CoA intermediates (Fig. 1). With the individual core/elongation components expressed from the chromosome under cumate-inducible control, candidate enzymes for alcohol production were cloned into pCDF to provide independent control of expression in JST07 (DE3). Alcohol-forming termination pathways explored for this purpose included bifunctional alcohol-forming acyl-CoA reductases, in which

a single enzyme converts the acyl-CoA to an alcohol, as well as a pathway in which multiple enzymes, an aldehyde-forming acyl-CoA reductase and alcohol dehydrogenase/aldehyde reductase, convert the acyl-CoA intermediate into the desired primary alcohol. The alcohol-forming acyl-CoA reductases AdhE2 from *Clostridium acetobutylicum* [9] and Maqu2507 [17, 35] and Maqu2220 [12, 17] from *Marinobacter aquaeolei* were selected for the single enzyme approach. For the first step of the multi-enzyme termination pathway, we chose the aldehyde-forming acyl-CoA/ACP reductases Acr1 from *Acinetobacter calcoaceticus* [27], AcrM from *Acinetobacter* sp. strain M-1 [13], Ald from *Clostridium beijerinckii* [33], and Orf1594 from *Synechococcus elongatus* PCC 7942 [31], as well as predicted acyl-CoA reductases EutE from *Salmonella typhimurium* [36] and MhpF from *E. coli* [8].

As seen in Table 3, when genes encoding these enzymes were expressed in JST07 (DE3) along with *bktB*, *fadB*, and *egTER*, varying amounts of C4–C10 primary alcohols were produced dependent on the enzymes utilized. Acyl-CoA reductases Acr1, AcrM, and Orf1594 did not produce alcohols shorter than 10 carbons in length, consistent with previous studies demonstrating preference for longer chain length substrates [13, 27, 31]. While the Clostridal enzymes AdhE2 and Ald are involved in butanol production in their native species, when these enzymes were expressed in a background enabling the generation of C6–C10 acyl-CoA intermediates, hexanol, octanol, and decanol were produced in addition to butanol (Table 3). While Ald expression resulted in significant amounts of butanol production, greater than 150 mg/L of C6–C10 alcohols were produced demonstrating the potential of this enzyme for medium-chain length primary alcohol production. Furthermore, of all candidate enzymes tested, the alcohol-forming acyl-CoA reductase Maqu2507 from *M. aquaeolei* produced



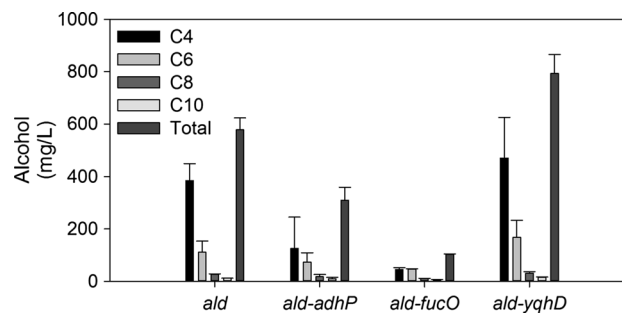
**Table 3** Primary C4–C10 alcohol production in JST07 (DE3) with functional  $\beta$ -oxidation reversal (BktB, FadB, *eg*TER) from chromosomal expression and indicated acyl-CoA reductase expression from pCDF vector

Acyl-CoA reductase	Primary alcohol titer (mg/L)			
	Butanol	Hexanol	Octanol	Decanol
<i>acr1</i>	0	0	0	26 $\pm$ 0.5
<i>acrM</i>	0	0	0	12 $\pm$ 12
<i>adhE2</i>	25 $\pm$ 25	54 $\pm$ 0.5	40 $\pm$ 0.5	27 $\pm$ 0.5
<i>Ald</i>	385 $\pm$ 15	81 $\pm$ 0.5	47 $\pm$ 0.5	27 $\pm$ 0.5
<i>eutE</i>	195 $\pm$ 5	65 $\pm$ 1	45 $\pm$ 0.5	28 $\pm$ 0.5
<i>mhpF</i>	0	50 $\pm$ 0.5	0	0
<i>maqu2220</i>	0	0	0	0
<i>maqu2507</i>	85 $\pm$ 15	105 $\pm$ 8	59 $\pm$ 3	39 $\pm$ 0.5
<i>orf1594</i>	0	0	0	25 $\pm$ 0.5

the highest titer (~200 mg/L) of C6–C10 primary alcohols, while also limiting the amount of butanol produced in relation to these target chain lengths (Table 3).

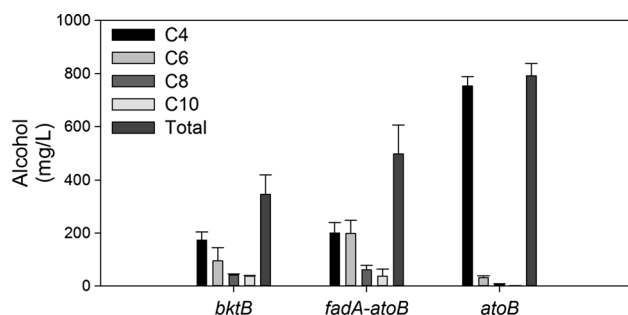
Given the higher titers of C6–C10 primary alcohols with the use of Ald and Maqu2507, and the potential for acyl-CoA reductases to act on acyl-ACP intermediates from the fatty acid biosynthesis pathway, experiments were run to confirm the production of these alcohols were a result of a functional  $\beta$ -oxidation reversal. Alcohol production with Ald and Maqu2507 was only observed when combined with a full  $\beta$ -oxidation reversal (BktB, FadB, and *eg*TER), with no alcohol production detected with the expression of either enzyme in JST07 (DE3) without all core/elongation enzymes (data not shown). Based on these results, Ald and Maqu2507 were selected as the termination pathways for further investigation into alcohol production from a  $\beta$ -oxidation reversal.

One interesting facet of the use of Ald for acyl-CoA reduction is that despite Ald representing an aldehyde-forming acyl-CoA reductase, as opposed to a bi-functional alcohol-forming enzyme, alcohol production was observed without the overexpression of an alcohol dehydrogenase/aldehyde reductase. This is likely because *E. coli* possesses numerous aldehyde reductases that can reduce varying chain length aldehydes to the corresponding alcohols [26, 29]. While this can explain alcohol production in the case of the aldehyde-forming acyl-CoA reductase expression, it also represents a potential control point for increasing the titer and chain length specificity of the primary alcohols formed. This was further explored by the combined overexpression of *ald* with several native aldehyde reductases/alcohol dehydrogenases. These included FucO and YqhD, previously demonstrated to function for butanol production from a  $\beta$ -oxidation reversal [6], as well as AdhP, an enzyme recently shown to be involved in the reduction of aldehydes to primary alcohols in an engineered *E. coli* strain [19].

**Fig. 5** Primary alcohol production from a  $\beta$ -oxidation reversal with the expression of the aldehyde-forming acyl-CoA reductase Ald from *C. beijerinckii* individually or in combination with the *E. coli* alcohol dehydrogenases AdhP, FucO, or YqhD. Termination pathway enzyme(s) expressed from pCDF in JST07 (DE3) containing constructs for *bktB*, *fadB*, and *eg*TER chromosomal expression

The genes encoding these enzymes were cloned into pCDF-P1-*ald* behind the second T7 promoter for expression in JST07 (DE3) with chromosomal expression of *bktB*, *fadB*, and *eg*TER. Interestingly, while the co-expression of *adhP* or *fucO* with *ald* had neutral to negative impact on alcohol production, *yqhD* overexpression with *ald* resulted in an overall increase to C4–C10 alcohol production (Fig. 5). This increase in total titer was associated with higher levels of butanol and hexanol production, indicating that YqhD could contribute to the conversion of C4–C6 aldehydes to the respective alcohol.

In contrast to Ald, the bi-functional alcohol-forming acyl-CoA reductase Maqu2507 from *M. aquaeolei* is capable of fully reducing an acyl-CoA intermediate to the primary alcohol, likely negating any potential influence of endogenous alcohol dehydrogenases on target product synthesis. However, the broad chain length specificity of this enzyme displayed within the context of a  $\beta$ -oxidation reversal (Table 3) provides the opportunity to dictate product chain length through control of the chain length of acyl-CoA intermediates generated. As previously demonstrated with carboxylic acid synthesis (Fig. 3), the combination of various thiolases with different termination pathways can greatly influence the product chain length distribution depending on the key characteristics of the enzymes utilized. Using a similar approach with Maqu2507, we sought to determine the influence the use of various thiolases had on alcohol production. For this purpose, the pCDF vector expressing the gene encoding Maqu2507 was transformed into the JST07 (DE3) containing chromosomal expression constructs for FadB and *eg*TER in combination with the construct(s) for AtoB, BktB, or AtoB and FadA. As seen in Fig. 6, when these various thiolase(s) are utilized as the condensation enzyme, the chain length of alcohols varies according to the chain length specificity of the thiolase.



**Fig. 6** Controlling primary alcohol chain length from a  $\beta$ -oxidation reversal through the use of various thiolase modules. Maqu2507 from *M. aquaeolei* (expressed from pCDF-P1-*maqu2507*) was used for termination in JST07 (DE3) containing chromosomal constructs for the expression of *fadB*, *egTER*, and the indicated thiolase(s)

As expected based on the kinetic properties of AtoB, when this enzyme is used as the exclusive thiolytic component, butanol is the predominant alcohol produced. However, when AtoB is used in conjunction with FadA, the distribution of chain length produced shifts to longer chain length alcohols with C6–C10 alcohols accounting for the highest product fraction (Fig. 6). Interestingly, unlike the case of carboxylic acid production with *tesA'* expression, in which BktB is enabled in the highest titers (Fig. 3), with Maqu2507 as the termination pathway the combination of AtoB and FadA enables the higher total titers of targeted C6–C10 products (296 mg/L) (Fig. 6). This could be reflective of the relative kinetic properties of the thiolase(s) and termination enzymes in regard to the acyl-CoA intermediates. Considering the acyl-CoA intermediate(s) represent the substrate for both the condensation enzyme(s) as well as the termination pathways to saturated carboxylic acids and primary alcohols in this case, the competition between these enzymes for varying chain length acyl-CoA intermediates represents a key element in determining the distribution of products. While the kinetic properties of these enzymes for the acyl-CoA chain lengths in question have not been determined, one can envision a scenario in which the relative substrate affinities and catalytic properties of the thiolase(s) and termination enzymes dictate the distribution of products based on the overall competition for the acyl-CoA intermediates. As such, the continued refinement and identification and characterization of core/elongation and termination pathways with favorable kinetic properties can be utilized to improve both the overall titer and product selectivity from a  $\beta$ -oxidation reversal.

## Conclusions

Expanding upon the synthetic approach to reconstructing a functional  $\beta$ -oxidation reversal, we have expanded the utility of this pathway for the synthesis of medium-chain

length (C6–C10) products. This was accomplished through manipulation of the thiolase and termination components of the pathway to increase the generation of medium-chain length acyl-CoA intermediates and enable their conversion to products of interest. Key to this approach was the deletion of endogenous thioesterases, which imparted broad chain length specificity for acyl-CoA hydrolysis and resulted in the depletion of generated acyl-CoA intermediates to carboxylic acids. In this efficient background, the expression of thiolases of varying chain length specificity, along with all core  $\beta$ -oxidation modules and controlled thioesterase termination pathways altered the chain length distribution of carboxylic acid production. Titrers of about 1.3 g/L C6–C10 saturated carboxylic acids (hexanoic, octanoic, and decanoic acids) were observed with the use of BktB as the thiolase component and *TesA'* as the thioesterase termination pathway, representing the highest titers of this chain length distribution reported to date. Product functionality was tuned through the expression of various aldehyde/alcohol-forming acyl-CoA reductases in this background, resulting in the synthesis of C6–C10 primary alcohols from a  $\beta$ -oxidation reversal. The highest total titers of medium-chain primary alcohols hexanol, octanol, and decanol (~0.3 g/L) were achieved with the combination of AtoB and FadA as the thiolase components with the alcohol-forming acyl-CoA reductase Maqu2507 from *M. aquaeolei*. These results demonstrate both the ability to fine-tune product synthesis as well as the importance of selecting appropriate core/elongation and termination enzymes based on the targeted products. Furthermore, the feedstock-independent nature of the pathway (i.e., only requires the generation of acetyl-CoA from a given carbon source) was demonstrated through the use of the low-value feedstock glycerol as the carbon source. Overall, the results reported here further establish the  $\beta$ -oxidation pathway as a modular and versatile pathway that can be utilized for the synthesis of a wide array of fuels and chemicals.

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