Published in final edited form as:

Metab Eng. 2013 November ; 20: . doi:10.1016/j.ymben.2013.10.006.

Production of medium chain length fatty alcohols from glucose in *Escherichia coli*

J. Tyler Youngquist^{a,b}, Martin H. Schumacher^a, Joshua P. Rose^a, Thomas C. Raines^a, Mark C. Politz^a, Matthew F. Copeland^a, and Brian F. Pfleger^{a,b,*}

^a Department of Chemical and Biological Engineering, University of Wisconsin-Madison, Madison, WI 53706, United States

^b Great Lakes Bioenergy Research Center, University of Wisconsin-Madison, Madison, WI 53706, United States

Abstract

Metabolic engineering offers the opportunity to produce a wide range of commodity chemicals that are currently derived from petroleum or other non-renewable resources. Microbial synthesis of fatty alcohols is an attractive process because it can control the distribution of chain lengths and utilize low cost fermentation substrates. Specifically, primary alcohols with chain lengths of 12 to 14 carbons have many uses in the production of detergents, surfactants, and personal care products. The current challenge is to produce these compounds at titers and yields that would make them economically competitive. Here, we demonstrate a metabolic engineering strategy for producing fatty alcohols from glucose. To produce a high level of 1-dodecanol and 1-tetradecanol, an acyl-ACP thioesterase (BTE), an acyl-CoA ligase (FadD), and an acyl-CoA/aldehyde reductase (MAACR) were overexpressed in an engineered strain of *Escherichia coli*. Yields were improved by balancing expression levels of each gene, using a fed-batch cultivation strategy, and adding a solvent to the culture for extracting the product from cells. Using these strategies, a titer of over 1.6 g/L fatty alcohol with a yield of over 0.13 g fatty alcohol / g carbon source was achieved. These are the highest reported yield of fatty alcohols produced from glucose in *E. coli*.

Keywords

E. coli; thioesterase; acyl-CoA reductase; fatty alcohol; dodecanol; tetradecanol

Introduction

The finite nature of fossil fuels, as well as rising prices and environmental concerns, has spurred research to develop chemical production alternatives that are more sustainable. One such alternative is to use engineered microorganisms to convert renewable growth substrates (e.g. sugars) to metabolic products of interest. Using modern genetic techniques and synthetic biology approaches, microorganisms have been engineered to produce a wide variety of chemicals from renewable starting materials (Keasling 2012; Dellomonaco et al.,

© 2013 Elsevier Inc. All rights reserved

* Corresponding author. 3629 Engineering Hall, 1415 Engineering Drive, Madison, WI 53706, United States. Phone: +1 608 890 1940. Fax: +1 608 262-5434. pfleger@engr.wisc.edu..

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

2010). Metabolic engineering offers the ability to tailor the flow of carbon to desired compounds and leverage the advantages of enzymatic biocatalysts (e.g. specificity, precision, complexity). If economic and productivity targets can be met, engineered microbes could play a large role in replacing the fraction of petroleum used to produce the chemical building blocks that enable current lifestyles.

In recent years, significant effort has focused on producing hydrophobic compounds via fatty acid biosynthesis for use as liquid transportation fuels or commodity chemicals (Lennen and Pfleger 2013). Aliphatic compounds such as fatty alcohols also have applications as detergents, emulsifiers, lubricants, and cosmetics. While fatty alcohols normally make up about 3-5 percent of the final formulation of these products, some such as solid anti-perspirants contain up to 25% fatty alcohols (Mudge et al., 2008). As of 2006, over 1.3 million tons of fatty alcohols were used worldwide each year (Mudge et al., 2008). As a whole, the industry represents over a 3 billion dollar market (Rupilius and Ahmad, 2006). Currently, fatty alcohols are produced either through processing natural fats and oils (oleochemicals) or from petrochemicals (e.g. crude oil, natural gas). In the oleochemical route, fatty acids or fatty acid methyl esters are released from triglycerides and hydrogenated to form fatty alcohols (Matheson 1996). In one common petrochemical route, paraffins are separated from kerosene, then converted to olefins, before being converted to fatty alcohols. As both processes require either modifications to biodiesel or petrochemical fuel stocks, microbial production of fatty alcohols from renewable sugars is a promising alternative.

Fatty alcohols can be generated by microorganisms endogenously (**Fig. 1a**) via reduction of fatty aldehydes that are made via reduction of acyl-thioesters (coenzyme A or acyl-carrier protein) (Reiser and Somerville 1997). Alternatively, fatty acids have been shown to be directly converted to fatty aldehydes via the action of a carboxylic acid reductase (Akhtar et al., 2013). Genes encoding long chain acyl-CoA reductase activity have been isolated from many organisms including bacteria (Reiser and Somerville 1997), insects (Linard et al., 2010), birds (Hellenbrand et al., 2011), mammals (Cheng and Russell 2004), and protists (Teerawanichpan and Qiu, 2010). Many of these enzymes are used to synthesize fatty alcohols as precursors to wax esters. Three classes of reductases have been expressed in *E. coli* to produce C12-14 alcohols – reductases from soil bacteria (Reiser and Somerville, 1997; Steen et al., 2010), reductases from plants such as *Arabidopsis* or *Simmondsia* (Doan et al., 2009; Rowland and Domergue, 2012), and reductases found in marine bacteria (Willis et al., 2011; Hofvander et al., 2011). These classes differ in their ability to catalyze multiple reactions and in their substrate preference. Reductases similar to those found in *Acinetobacter* contain only the domain to catalyze conversion of acyl-thioesters to fatty aldehydes. Conversely, reductases from plants can catalyze both reductions, but generally do not have broad substrate specificity, preferring the dominant long acyl chains found in lipids. Reductases from marine bacteria catalyze both reductions and are active on a wide range of chain lengths.

While fatty acids have been produced with yields of greater than 0.2 g fatty acid per gram carbon source consumed (Dellomonaco et al., 2011; Zhang et al., 2012), the highest reported yields of fatty alcohols have been at least five fold lower. The work of Steen et al (Steen et al., 2010) demonstrated that fatty alcohols can be produced through overexpression of a thioesterase (*tesA*), an acyl-CoA ligase (*fadD*), and an acyl-CoA reductase (*acrI*), with titers of around 60 mg/L fatty alcohol and yields of less than 0.005 g fatty alcohol / g carbon source. Further metabolic engineering and fermentation efforts have increased the titer to ~450 mg/L, but with no significant improvement in yield (Zheng et al., 2012). Alternative strategies have led to slightly higher fatty alcohol yields from a defined carbon source. Expressing a carboxylic acid reductase and aldehyde reductase, titers have reached ~350 mg/L with a yield of 0.04 g fatty alcohol / g carbon source (Akhtar et al., 2013). Through the

reversal of the fatty acid β -oxidation, yields have been achieved between 0.04 and 0.055 g fatty alcohol / g carbon source consumed (Dellomonaco et al., 2011). However, no published strategy has yet to focus on the individual tailoring of expression for the multiple enzymes required in medium chain length fatty alcohol production.

In this work, we present a strategy to improve the yield of 1-dodecanol and 1-tetradecanol from an unrelated carbon source (e.g. glucose) in *E. coli*. We characterized three acyl-CoA ligase and three acyl-CoA reductase enzymes in conjunction with the thioesterase BTE from *Umbellularia californica* to determine the impact on fatty acid consumption and fatty alcohol synthesis. Additionally, we altered individual expression levels of the thioesterase, acyl-CoA ligase, and acyl-CoA reductase to best convert sugar substrates into fatty alcohol products. Our final strain overexpressed BTE, native FadD from *E. coli*, and the acyl-CoA reductase (MAACR) from *Marinobacter aquaeolei* VT8. In a bioreactor, a titer of over 1.65 g/L fatty alcohol (1.55 g/L C12-14 alcohol) and a yield of over 0.13 g fatty alcohol / g consumed glucose (0.12 g C12-14 fatty alcohol / g consumed glucose) was achieved.

2. Materials and Methods

2.1. Bacterial strains and chromosome engineering

All bacterial strains used in this study are listed in **Table 1**. Single gene deletions were transferred P1 transduction of phage lysates from the collection of single gene knockouts from the National BioResource Project (NIG, Japan) (Baba et al. 2006). Chromosomal integration of a BTE expression cassette (acyl-ACP thioesterase from *Umbellularia californica* under the control of the IPTG inducible P_{trc} promoter) was performed as described previously (Youngquist et al. 2012). All deletions and insertions were verified by colony PCR.

2.2. Reagents and media

Enzymes were purchased from New England Biolabs (Ipswich, MA). Nucleic acid purification materials were purchased from Qiagen (Venlo, Netherlands), Promega (Madison, WI), or Thermo Scientific (Waltham, MA). Chemicals were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Hampton, NH) unless otherwise specified. Oligonucleotides (sequences are listed in **Table 2**) were purchased from Integrated DNA Technologies (Coralville, IA). For all growth experiments, single colonies obtained from freezer stocks were used to inoculate 5 mL LB starter cultures grown overnight prior to the inoculation of experimental cultures. All shake flask growth experiments were performed at 30°C in a rotary shaker (250 rpm). Cultures were supplemented with appropriate antibiotics (100 $\mu\text{g mL}^{-1}$ ampicillin and/or 50 $\mu\text{g mL}^{-1}$ kanamycin and/or 34 $\mu\text{g mL}^{-1}$ chloramphenicol) where necessary.

2.3. Plasmid Construction

All plasmids used in this study are listed in **Table 1**. Enzyme encoding genes were cloned from native sources if each had been successfully expressed in *E. coli* at 30°C. If not, codon-optimized variants were custom synthesized. *E. coli* acyl-CoA synthetase *fadD* was amplified by PCR from genomic DNA isolated from *E. coli* MG1655. Codon optimized versions of the acyl-CoA synthetase *fadD6* (Accession number: WP_003900292), acyl-CoA reductase *acr1* (Accession number: P94129), and acyl-CoA reductase *far6* (Accession number: B9TSP7) were custom synthesized by Life Technologies (Carlsbad, CA). *P. putida* KT2440 genomic DNA was used as a template to PCR amplify PP_0763 (Accession number: NP_742924). MAACR (Accession number: A1U3L3) was amplified by PCR from a plasmid containing the *Marinobacter aquaeolei* acyl-CoA reductase generously donated by Dr. Brett Barney (University of Minnesota). Base plasmids pBTRKtrc, pACYCtrc, and

pUCtrc were constructed by generating PCR products using primers 1 and 2 to amplify the antibiotic resistance marker and origin of replication from plasmids pBAD35, pBAD33, and pBAD34 (Lennen et al., 2010) respectively. Primers 3 and 4 were then used to amplify the multi-cloning site, P_{trc} promoter, and *lacI^q* region from pTrc99a. The PCR products were combined using the Gibson assembly method (Gibson et al. 2009). To construct each of the individual expression plasmids, pBTRKtrc, pACYCtrc, pTrc99a, and pUCtrc were amplified with primers 13 and 14, MAACR was amplified with primers 5 and 6. These PCR products were then combined using the Gibson assembly method to generate the constructs listed in **Table 1**. For the codon optimized genes *acr1* and *far6*, pTrc99a and the vectors containing the genes were digested with *Kpn I* and *Hind III*, ligated with an analogous digest of pTrc99a using T4 DNA ligase. The same procedure was used with the codon optimized *fadD6* and pACYCtrc. For the other CoA synthetases, the PCR products from *fadD* (7 and 8) and PP_0763 (11 and 12) were digested with *Kpn I* and *Xba I*, and ligated with digested pACYCtrc. All constructs were confirmed by DNA sequencing.

2.4. Culturing conditions

For experiments where dodecanoic acid was supplied exogenously (**Fig. 1, 2, 3**), each strain was cultured in 50 mL LB starting with an inoculum at optical density (OD_{600}) of 0.02. At OD_{600} 0.2, cultures were induced with 1 mM isopropyl β -D-thiogalactopyranoside (IPTG) and supplemented with either 40 (for alcohol production studies) or 50 μ L (for dodecanoic acid consumption studies) of a 250 mg/mL solution of dodecanoic acid in ethanol (initial [dodecanoic acid] = 200 or 250 mg/L). After induction, cultures were incubated at 30°C with shaking and 2.5 mL culture samples were taken at either 2.5, 7, 9, and 11 or 4, 8, 12, and 24 hours after induction for dodecanoic acid consumption and alcohol production studies, respectively. Culture samples were processed for FAME analysis as described previously (Agnew et al. 2012).

For fatty alcohol production experiments (**Fig. 4, 5**), each strain was inoculated to an OD_{600} of 0.02 in 50 mL LB + 0.4% glycerol and induced with 1 mM IPTG at an OD_{600} of 0.2. Following induction, cultures were incubated with shaking at 30°C for 48 hours. Culture samples of 2.5 mL were taken at 24 and 48 hours for fatty alcohol and FAME analysis. To determine the fraction of fatty alcohol associated with cells, an additional 10 mL sample from the 48 hour timepoint was centrifuged at $4000 \times g$ for 10 min and the resulting cell pellet was resuspended to 10 mL in $1 \times$ PBS. After repeating the process, 2.5 mL of the resuspended cell pellet was taken for fatty alcohol and FAME analysis.

Bioreactor experiments were performed in a 3-L stirred bioreactor (Applikon Biotechnology, Inc., Schiedam, Netherlands), using a 1 L working volume. Temperature was maintained at 30°C using a heat blanket (Applikon, model number M3414) and cooling water. Reactor temperature, pH and dissolved oxygen (DO_2) were monitored using specific probes (Applikon). Carbon dioxide and oxygen off-gas levels were monitored using a Blusens BlueInOne Ferm (Blusens, Herten, Germany). Reactor pH was maintained at 7.00 ± 0.01 by the addition of 10% (v/v) NH_4OH or 1 M HCl solutions. Agitation was provided by a single impeller with the stir speed set between 240-320 rpm. Stirrer speed was varied to ensure the DO_2 content did not decrease below 40% saturation in order to maintain an aerobic environment (Becker et al., 1997; Tseng et al., 1996). The air inflow rate was maintained at 1.0 L/min.

Bioreactor experiments were performed using a phosphate limited MOPS minimal media recipe (Youngquist et al., 2013). Cultures were inoculated to an OD_{600} of 0.04 using a culture of *E. coli* MHS03, TY30, or TY34 containing pBTRK-MAACR grown to an $OD_{600} > 2$ in MOPS minimal media (Neidhardt et al., 1974) supplemented with 0.7% glucose overnight. Bioreactor starting media was MOPS minimal media supplemented with 0.7%

glucose, 0.276 mM potassium sulfate, and 9.5 mM ammonium chloride but containing only 370 μ M K_2HPO_4 . Cultures were induced with 1 mM IPTG at OD₆₀₀ 0.2. Each experiment was performed using a discontinuous fed-batch where a bolus of 2 g glucose (10 mL of a 20% (w/v) glucose solution) was added at 18, 24, 30, 42, and 48 hours post-induction. In three experiments, 20 mL dodecane was added to the culture 6 hours after induction to provide a sink for fatty alcohols. For all experiments, CO₂ off-gas levels and pH were measured continuously and culture samples (10 mL) were taken periodically prior to glucose additions to determine OD₆₀₀, as well as the concentrations of glucose, acetate, fatty alcohols, and fatty acids.

2.5. Fatty acid and fatty alcohol extraction and characterization

FAME analysis was performed on 2.5 mL of culture, supernatant, or resuspended washed cell pellet as described previously (Lennen et al., 2010). Analysis of fatty alcohols followed the same procedure except 20 μ L of 10 mg/mL pentadecanol in ethanol was added to the chloroform methanol mix as an internal standard in addition to the fatty acid internal standards.

To quantify plasmid copy number (Figure 4) cells were collected (500 μ L) at OD₆₀₀ 0.4 as well as at 24 hours post-induction. Collected cells were centrifuged at 16,000 $\times$ g for 1 minute, snap frozen in liquid nitrogen, and stored at -80°C. In preparation for quantitative PCR, cell pellets were resuspended in 50 μ L of nuclease-free water for the 0.4 OD₆₀₀ samples and 500 μ L of water for the 24 hour samples. One microliter of cell suspension was used directly in a quantitative PCR reaction using Bio-Rad iQ SYBR green supermix (Bio-Rad, Hercules, CA). Primers were used for amplifying plasmid based MAACR and chromosomal *ompA*. SYBR green fluorescence was measured over time with a CFX real-time thermocycler (Bio-Rad). Threshold cycle (C_t) values were calculated by regression analysis using Bio-Rad CFX manager software. Plasmid copy numbers of experimental samples were determined by establishing a standard curve for both the MAACR and *ompA* genes using purified pUC19-MAACR and TY30 genomic DNA, respectively.

For RNA samples, 1 mL of culture at an OD₆₀₀ of 0.8 was centrifuged at 8000 $\times$ g for 3 minutes at 4°C. The supernatant was quickly removed and then the cell pellet was snap frozen in a dry ice ethanol bath for 5 minutes before storing the samples at -80°C until further processing. RNA was isolated using an RNeasy mini kit (QIAGEN). Residual DNA was digested using the Ambion DNA-free™ Kit (Applied Biosystems). The corresponding cDNA was synthesized using the GoScript™ Reverse Transcription System (Promega) following manufacturer's instructions. To run the qPCR, the Maxima SYBR Green/Fluorescein qPCR Master Mix (Thermo Scientific) was used. Primers were designed for amplifying both a 100 bp region of *fadD* and a 100 bp region of *rrsA* to act as a reference for normalization of samples (Kobayashi et al., 2006).

3. Results

3.1. Establishing production of 1-dodecanol in *E. coli*

Fatty alcohol production was established in *E. coli* by heterologous expression of enzymes that catalyze reduction of acyl-thioesters and the resulting fatty aldehydes. In addition, two modifications of the *E. coli* MG1655 chromosome were necessary to produce 1-dodecanol from exogenously fed dodecanoic acid (Fig. 1b). First, β -oxidation was blocked to prevent consumption of the exogenously fed free fatty acid. Small amounts of 1-dodecanol was produced when this objective was accomplished by deleting *fadE* (encoding enoyl-CoA reductase). Second, acyl-CoA synthetase (FadD) activity was essential for converting the exogenous lauric acid to the corresponding acyl-CoA thioester, a substrate for the

heterologously expressed acyl-CoA/ACP reductase (i.e. MAACR from *Marinobacter aquaeolei* VT8 in **Fig. 1b**). Unexpectedly, the $\Delta fadE$ strain only converted 18% of the lauric acid fed to the culture. The fractional conversion of lauric acid to 1-dodecanol was increased when the levels of FadD were elevated by replacing the native P_{fadD} with the strong, IPTG inducible P_{trc} promoter. In all strains, small amounts of 1-hexadecanol (15-20% of the endogenous hexadecanoic acid content) were produced, demonstrating the activity of MAACR towards both native C_{16} acyl-ACPs and C_{12} -acyl-CoAs derived from exogenous lauric acid.

3.2. Impact of various acyl-CoA synthetase on consumption of lauric acid

Given the dependence of 1-dodecanol conversion on acyl-CoA synthetase activity, the impact of three candidate synthetases on dodecanol production was examined by determining the rates of lauric acid consumption in *E. coli* MHS04 ($\Delta fadD$, $\Delta fadR$). Deletion of *fadR* removed repression of enzymes involved in β -oxidation (Dirusso et al., 1992) and increased the likelihood that acyl-CoA synthesis was the rate limiting step in lauric acid consumption. FadD and two alternative acyl-CoA synthetases were cloned into a medium copy plasmid and expressed from the P_{trc} promoter. The second acyl-CoA synthetase gene, *fadD6* from *M. Tuberculosis* was chosen because it has high activity toward C_{12} fatty acids and is soluble even when highly expressed (Arora et al., 2005). A third acyl-CoA synthetase, PP_0763 from *Pseudomonas putida*, was selected because of its ability to activate C_{12} fatty acids and enhance medium chain length PHA production (Agnew et al., 2012; Wang et al., 2012). While each of the CoA synthetases conferred the ability to consume 250 mg/L lauric acid within 12 hours (**Fig. 2**), the strain expressing *fadD* was able to consume over 90% of the fed fatty acid within 8 hours. Each of the other ligases took at least 11 hours to reach the same mark. Based on this data, FadD was selected as the acyl-CoA synthase for the fatty alcohol production pathway.

3.3. Selection of acyl-CoA reductase

Once *fadD* was selected as the acyl-CoA ligase, it became necessary to determine the acyl-CoA reductase best suited for the conversion of C_{12} acyl-CoAs into fatty alcohols. Genes coding for three different types of acyl-CoA reductases (*acr1* from *Acinetobacter calcoaceticus* (Reiser and Somerville 1997), *far6* from *Arabidopsis thaliana* (Doan et al. 2009), and MAACR from *Marinobacter aquaeolei* VT8 (Willis et al., 2011)) were tested to see which allowed for the highest conversion of free fatty acids to fatty alcohols. Heterologous expression of a codon-optimized variant of *far6* failed to produce 1-dodecanol when cultures were fed dodecanoic acid (data not shown). Conversely, heterologous expression of both *acr1* and MAACR resulted in conversion of exogenous dodecanoic acid to 1-dodecanol. In these experiments, acyl-CoA reductases were expressed from medium copy plasmids harboring the IPTG inducible P_{trc} promoter in strain MHS01 ($\Delta fadE$ $\Phi[P_{Trc-fadD}]$). MAACR facilitated the fastest conversion of dodecanoic acid to 1-dodecanol (**Fig. 3**), with 80% of the initial fed dodecanoic acid had been converted to dodecanol within 12 hours after induction. One advantage of MAACR is its ability to also reduce dodecanaldehyde, by-passing endogenous aldehyde reductase activity and minimizing production of potentially toxic intermediates. Thus, MAACR was chosen as the acyl-CoA reductase for future alcohol production experiments.

3.4. Determining optimal expression levels of acyl-CoA synthesis and reduction under conditions of endogenous fatty acid production

In order to use sugars as a feedstock for alcohol production, the medium chain length thioesterase (BTE) from *Umbellularia californica* (Voelker and Davies 1994) was heterologously expressed in *E. coli* to endogenously produce C_{12} and C_{14} free fatty acids for

subsequent conversion to the corresponding alcohols. A family of fatty acid producing strains were constructed by inserting a DNA cassette containing BTE under the control of the IPTG inducible P_{trc} promoter into various genomic loci (*fadE*, *fadAB*, and *ackA-pta*). Increasing BTE copy number (up to 3 copies) has been shown to increase free fatty acid titers (Youngquist et al. 2012). In effort to balance the expression of the downstream reductive activities with fatty acid production, the acyl-CoA reductase from *M. aquaeolei*, MAACR, was cloned onto a series of plasmids (origins of replication: pBBR1, pACYC, pBR322, and pUC) that were determined to have copy numbers of 1.74 ± 0.12 , 7.26 ± 1.33 , 14.52 ± 1.93 , and 56.37 ± 19.94 (relative to *ompA*) at OD₆₀₀ of 0.4. Each MAACR plasmid was expressed in either MHS03 (1× BTE) or TY30 (2× BTE) to identify the optimal level of gene expression for each activity. Each strain contained elevated acyl-CoA synthetase activity in the form of a P_{trc} -*fadD* chromosomal cassette.

Strains expressing MAACR from the low copy number pBBR1 origin plasmid produced the most fatty alcohols (**Fig. 4**), while strains with the high copy number pUC origin plasmid produced the least. Additionally, the strains containing the pUC origin plasmid displayed significantly impaired growth compared to the other strains (data not shown), suggesting a high metabolic burden associated with over-expression of MAACR. Surprisingly, there was small difference in final fatty alcohol titer between the same plasmid expressed in either the MHS03 or TY30 strain.

To optimize the level of acyl-CoA synthetase activity, a family of strains was constructed to vary *fadD* expression. *E. coli* TY33 ($\Delta fadD$), TY31 (native *fadD*), and TY30 (ΦP_{trc} -*fadD*), were transformed with pBTRK-MAACR and either pACYC_{trc} or pACYC-*fadD*. Expression of *fadD* was quantified by qPCR using RNA samples isolated at an OD₆₀₀ of 0.8. The *fadD* promoter replacement resulted in the maximum production of both 1-dodecanol and 1-tetradecanol (**Fig. 5**). The promoter replacement increased *fadD* levels by 46 ± 10 fold while expression from a medium copy plasmid increased expression by 1000 ± 200 fold relative to *fadD* under its native promoter on the chromosome.

3.5. Endogenous production of dodecanol and tetradecanol from glucose

To determine fatty alcohol yield, strains MHS03 (1× BTE), TY30 (2× BTE), and TY34 (3× BTE) each containing pBTRK-MAACR were cultivated in MOPS minimal media using glucose as a carbon source in controlled bioreactors. To simulate a fed batch, a bolus of 2 g glucose was added on five separate occasions. After 120 hours the final fatty alcohol titer was 280, 470, and 1185 mg/L for the MHS03, TY30, and TY34 versions, respectively, with over 90% coming from 1-dodecanol and 1-tetradecanol (**Fig. 6**). Based on the amount of glucose consumed by these cultures, the resulting yields were 0.031, 0.040, and 0.097 g fatty alcohol per g glucose consumed for the MHS03, TY30, and TY34 strains expressing MAACR, respectively.

In each experiment, a slight white sludgy material (assumed to be fatty alcohol) was deposited on the bioreactor wall. This material prevented an accurate timecourse of fatty alcohol production from being taken. To by-pass the problem, 20 mL of dodecane was added to the fermentation 6-h after induction. Three replicates of TY34 containing pBTRK-MAACR were run in controlled bioreactor fermentations with the dodecane emulsion. The addition of dodecane allowed for an accurate timecourse of fatty alcohol production to be taken and increased the final fatty alcohol titers to 1.65 g/L (0.134 g alcohol/g glucose consumed, **Fig. 6a**). Samples were taken to monitor biomass, CO₂, acetate, free fatty acids, and other excreted metabolites (**Fig. 6b, 6c, 6d, 6f**). Analysis of these samples led to a carbon balance accounting for 86% of the carbon, with elevated levels of acetate and CO₂ being produced.

Separate samples for the supernatant and cell pellet were taken at the last time point of each bioreactor run to determine if the addition of dodecane allowed for an increased transport of fatty alcohols to the extracellular medium. Less than 5% of the free fatty acids and fatty alcohols were found in the cell pellet fraction from cultures grown in co-culture (**Fig. 6c**). In contrast approximately 60% of the fatty alcohol species were found in the cell pellet fraction.

4. Discussion

4.1. Selection of Acyl-CoA Reductases

The selection of an acyl-CoA reductase influences fatty alcohol production in multiple ways. Biosynthesis of specific chain length fatty alcohols requires cleavage and reduction of the corresponding acyl-thioester (-CoA or -ACP) to a fatty aldehyde. The distribution of chain lengths for most fatty alcohol producers matches the strain's fatty acid profile, indicating that the reductase activity/affinity is not strong (or at least weaker than that of fatty acid elongation) for shorter chain substrates. Conversely, thioesterases are known to have high activity on a wide range of acyl-thioester chain lengths depending on the specific enzyme. The disadvantage of utilizing thioesterases for fatty alcohol production is the need to reactivate the acyl-chain for reduction. If acyl-ACP reductases could be engineered to have stronger activities towards specific acyl-ACPs, higher yields could be achieved. Similar efforts to engineer chain length specificity in thioesterases has been reported in the patent literature (Yuan et al., 1999) and could guide acyl-ACP reductase engineering.

Here, expression of the dual-activity acyl-CoA reductase, MAACR, led to the highest fatty alcohol productivity. It is likely that substrate channeling between the acyl-ACP and fatty aldehyde reduction domains prevented release of the reactive, potentially toxic, aldehyde intermediate. If novel, high-activity acyl-CoA reductases are identified, fusion (or incorporation into a complex via a protein scaffold) of aldehyde reductases could have similar benefits (Dueber et al. 2009). Alternatively, separate enzymes could be targeted to microcompartments to sequester the aldehydes from the cytoplasm. Many bacteria use this strategy to avoid the toxicity of aldehyde intermediates and/or increase the local concentration of substrates when enzymes have weak activity (Sampson and Bobik 2008; Frank et al., 2013). While this strategy is promising, the microcompartments would need to be engineered to transport the substrates (e.g. acyl-ACP) and products.

4.2. Balancing Expression of CoA Synthetase and Acyl-CoA Reductase

One of the metabolic engineering objectives in this study was to tailor the expression levels of the acyl-CoA synthetase and acyl-CoA reductase to balance the overall conversion between fatty acid and fatty alcohol. The optimal levels of acyl-CoA synthetase and acyl-CoA reductase that maintain balanced activity could be determined from knowledge of the *in vivo* kinetic parameters (k_{cat} , K_m), if known. Based on *in vitro* experiments, the specific activity and K_m for *fadD* conversion of lauric acid to lauryl-CoA are 2,630 nmol/min/mg protein and 1.6 μ M, respectively (Kamedas and Nunn 1981). For the conversion of lauryl-CoA to the aldehyde intermediate, the specific activity of MAACR *in vitro* is 34 nmol/min/mg enzyme and the K_m is 4 μ M (the specific activity for the second step, aldehyde to alcohol, is two orders of magnitude higher) (Willis et al., 2011). These values suggest that the k_{cat}/K_m ratio is about 100 fold higher for the acyl-CoA synthetase step than the reductase step. Optimal production of fatty alcohols occurred with MAACR on a low copy (~2:1 ratio to genomic DNA) plasmid using the same promoter as the chromosomal P_{trc} -*fadD* cassette (Lennen et al., 2010). Overexpression of MAACR decreased fatty alcohol titer, placing an upper limit on acyl-CoA reductase activity. This observation could be attributed to metabolic burden of protein overexpression or improper folding of MAACR expressed at a

high level. Prior studies have shown that soluble overexpression of MAACR is problematic without addition of an N-terminal maltose binding protein (Willis et al., 2011), which was used in this study. **Figure 5** shows that a 45 fold decrease in *fadD* transcript levels, between that controlled by P_{trc} promoter and the native promoter, resulted in only a 50% decrease in fatty alcohol titer. This result suggests that further strain optimization could be achieved by decreasing *fadD* expression. This strategy is consistent with the optimal ratio of MAACR and FadD levels predicted by their relative *in vitro* kinetics.

4.3. Improving Fatty Alcohol Yield

Implementation of the metabolic engineering strategy described above generated a strain that produced the highest reported yield (0.134 g/g) and titer of fatty alcohols (1.65 g/L fatty alcohols, with 77% and 17% being C12 and C14 species, respectively) from glucose. Previous studies that leveraged native fatty acid biosynthesis pathways produced fatty alcohol titers of up to ~450 mg/L C12-14 fatty alcohols with yields less than 0.01 g fatty alcohol / g carbon source (Steen et al. 2010; Zheng et al. 2012). Alternative pathways in *E. coli* have yielded up to ~350 mg/L fatty alcohols and yields up to 0.05 g fatty alcohol / g carbon source (Akhtar et al., 2013; Dellomonaco et al., 2011). Based on theoretical yields, *E. coli* is capable of producing 0.32 g 1-dodecanol per g glucose fed. As current yields are much less than theoretical, further optimization and metabolic engineering efforts are needed to improve yield. However, current yields of combined C12-14 fatty acids and fatty alcohols are similar to that seen in a corresponding FFA producing strain (Youngquist et al., 2013), indicating that efforts should focus on redirecting carbon flux toward fatty acid biosynthesis (Lennen and Pfleger 2012) rather than the conversion to fatty alcohol. The carbon balance on the bioreactor (**Fig. 6**) indicates that a significant amount of fed carbon is going to carbon dioxide production. Therefore, decreasing flux to CO₂ production could lead to improved fatty alcohol titers. Similarly, a small percentage of carbon flux ended in the secretion of acetate (**Fig. 6**). Given that TY34 is $\Delta ackA\Delta pta$, it is likely that the observed acetate was generated by the pyruvate oxidation pathway that concurrently generates proton motive force, as it is coupled to the electron transport chain (Abdel-Hamid et al., 2001). Deletion of *poxB* would eliminate acetate production through this pathway and potentially increase fatty alcohol yields (Zha et al. 2009; Peng Xu et al. 2013). Other studies have successfully achieved higher yields of fatty acids by overexpressing genes *fabZ* (Ranganathan et al. 2012) or *fadR* (Zhang et al. 2012). Similar manipulations employed in conjunction with this metabolic engineering strategy could lead to fatty alcohol production at yields closer to the theoretical limit.

The high yield and titer reported above was achieved by cultivating strain *E. coli* TY34 pBTRK-MAACR in a 1-L working volume using a fed-batch strategy. One interesting observation from this experiment was the consistent production of fatty alcohols and consumption of glucose (**Fig. 6**) during a prolonged stationary phase (>96 hours). Such a result may be expected due to the unique qualities of phosphate starvation, in which metabolic activity in the cell remains high despite no further cell growth (Ballesteros et al., 2001). During stationary phase a specific productivity of 0.016 g fatty alcohol/gDCW/h was observed and a glucose consumption rate of 0.11 g glucose/gDCW/h. A better understanding of metabolism and regulation under these conditions will help guide efforts to maintain the stability of producing strains and maximize the time strains can spend in the production phase.

5. Conclusions

Escherichia coli was engineered to produce 1-dodecanol and 1-tetradecanol from glucose. Cultivation of the strain in a bioreactor with 10% dodecane achieved the highest reported

titer (1.65 g/L) and yield (0.134 g fatty alcohol / g glucose) from a minimal glucose based media to date. The key steps to optimize this fatty alcohol producing strain were selection of FadD from *E. coli* as the acyl-CoA synthetase and MAACR from *M. aquaeolei* VT8 as the acyl-CoA reductase. In addition, overexpression of these two enzymes was found to be detrimental to fatty alcohol productivity. The optimal expression levels were found by replacing the native P_{fadD} promoter with a stronger inducible promoter (P_{trc}) and expressing MAACR from a low copy vector. The yields observed were nearly equivalent to the yield of free fatty acids in past work (Youngquist et al, 2013), suggesting that the strain may be capable of higher yields if free fatty acid production could be increased. Future work will aim to reduce the flux to other products that compete with flux to fatty alcohols.

Acknowledgments

This work was funded by the DOE Great Lakes Bioenergy Research Center (GLBRC; DOE Office of Science BER DE-FC02-07ER64494) and the National Science Foundation (CBET-1149678). The authors are grateful to Brett Barney, Zachariah Harris, Mick McGee, and Daniel Mendez-Perez for their contributions.

Abbreviations

ACL	acyl CoA ligase
ACR	acyl CoA reductase
PCR	polymerase chain reaction
BTE	Umbellularia californica thioesterase
MAACR	Marinobacter <i>aquaeolei</i> VT8 ACR
GC/MS	Gas Chromatography Mass Spectrometry
C_x	fatty acid or alcohol species containing x number of carbon atoms

7. References

- Abdel-Hamid, a M.; Attwood, MM.; Guest, JR. Pyruvate oxidase contributes to the aerobic growth efficiency of *Escherichia coli*. Microbiology (Reading, England). 2001; 147:1483–98.
- Agnew DE, Stevermer AK, Youngquist JT, Pflieger BF. Engineering *Escherichia coli* for production of C12-C14 polyhydroxyalkanoate from glucose. Metab. Eng. 2012; 14:705–13. [PubMed: 23141473]
- Akhtar MK, Turner NJ, Jones PR. Carboxylic acid reductase is a versatile enzyme for the conversion of fatty acids into fuels and chemical commodities. Proc. Natl. Acad. Sci. U.S.A. 2013; 110:87–92. [PubMed: 23248280]
- Amann E, Ochs B, Abel K. Tightly regulated tac promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene. 1988; 69:301–315. [PubMed: 3069586]
- Arora P, Vats A, Saxena P, Mohanty D, Gokhale RS. Promiscuous fatty acyl CoA ligases produce acyl-CoA and acyl-SNAC precursors for polyketide biosynthesis. J. Am. Chem. Soc. 2005; 127:9388–9. [PubMed: 15984864]
- Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba. M, Datsenko KA, Tomita M, Wanner BL, Mori H. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. 2006; 2 2006.0008.
- Ballesteros M, Fredriksson Å, Henriksson J, Nyström T. Bacterial senescence: protein oxidation in non-proliferating cells is dictated by the accuracy of the ribosomes. The Eur. Mol. Biol. Organ. J. 2001; 20:5280–5289.
- Becker S, Vlad D, Schuster S, Pfeiffer P, Unden G. Regulatory O₂ tensions for the synthesis of fermentation products in *Escherichia coli* and relation to aerobic respiration. Arch. Microbiol. 1997; 168:290–296. [PubMed: 9297466]

- Cheng JB, Russell DW. Mammalian wax biosynthesis. I. Identification of two fatty acyl-Coenzyme A reductases with different substrate specificities and tissue distributions. *J. Biol. Chem.* 2004; 279:37789–97. [PubMed: 15220348]
- Dellomonaco C, Clomburg JM, Miller EN, Gonzalez R. Engineered reversal of the β -oxidation cycle for the synthesis of fuels and chemicals. *Nature.* 2011; 476:355–9. [PubMed: 21832992]
- Dellomonaco C, Fava F, Gonzalez R. The path to next generation biofuels: successes and challenges in the era of synthetic biology. *Microb. Cell Fact.* 2010; 9:3. [PubMed: 20089184]
- Dirusso CC, Heimert TL, Metzger AK. Characterization of FadR , a Global Transcriptional Regulator of Fatty Acid Metabolism in *Escherichia coli*. *J. Biol. Chem.* 1992; 267:8685–8691. [PubMed: 1569108]
- Doan TTP, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P. Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. *J. Plant Physiol.* 2009; 166:787–796. [PubMed: 19062129]
- Dueber JE, Wu GC, Malmirchegini GR, Moon TS, Petzold CJ, Ullal AV, Prather KLJ, Keasling JD. Synthetic protein scaffolds provide modular control over metabolic flux. *Nat. Biotechnol.* 2009; 27:753–9. [PubMed: 19648908]
- Frank S, Lawrence AD, Prentice MB, Warren MJ. Bacterial microcompartments moving into a synthetic biological world. *J. Biotechnol.* 2013; 163:273–9. [PubMed: 22982517]
- Gibson DG, Young L, Chuang R-Y, Venter JC, Hutchison CA, Smith HO. Enzymatic assembly of DNA molecules up to several hundred kilobases. *Nat. Methods.* 2009; 6:343–345. [PubMed: 19363495]
- Hellenbrand J, Biester E-M, Gruber J, Hamberg M, Frentzen M. Fatty acyl-CoA reductases of birds. *BMC Biochem.* 2011; 12:64. [PubMed: 22151413]
- Hofvander P, Doan TTP, Hamberg M. A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. *FEBS Lett.* 2011; 585:3538–43. [PubMed: 22020216]
- Kamedas K, Nunn WD. Purification and Characterization of Acyl Coenzyme A Synthetase from *Escherichia coli*. *J. Biol. Chem.* 1981; 256:5702–5707. [PubMed: 7016858]
- Keasling JD. Synthetic biology and the development of tools for metabolic engineering. *Metab. Eng.* 2012; 14:189–95. [PubMed: 22314049]
- Kobayashi A, Hirakawa H, Hirata T, Nishino K, Yamaguchi A. Growth phase-dependent expression of drug exporters in *Escherichia coli* and its contribution to drug tolerance. *J. Bacteriol.* 2006; 188:5693–703. [PubMed: 16885437]
- Lennen RM, Braden DJ, West RA, Dumesic JA, Pfleger BF. A process for microbial hydrocarbon synthesis: Overproduction of fatty acids in *Escherichia coli* and catalytic conversion to alkanes. *Biotechnol. Bioeng.* 2010; 106:193–202. [PubMed: 20073090]
- Lennen RM, Pfleger BF. Engineering *Escherichia coli* to synthesize free fatty acids. *Trends Biotechnol.* 2012; 30:659–67. [PubMed: 23102412]
- Lennen RM, Pfleger BF. Microbial production of fatty acid-derived fuels and chemicals. *Curr. Opin. Biotechnol.* 2013; 1–10. [PubMed: 23273659]
- Liénard MA, Hagström AK, Lassance J-M, Löfstedt C. Evolution of multicomponent pheromone signals in small ermine moths involves a single fatty-acyl reductase gene. *Proc. Natl. Acad. Sci. U.S.A.* 2010; 107:10955–60. [PubMed: 20534481]
- Matheson, KL. Surfactants raw materials: classification, synthesis, and uses. In: Spitz, L., editor. *Soaps and Detergents: A Theoretical and Practical Review*. AOCS; Champaign, IL: 1996. p. 288-303.
- Mudge, SM.; Belanger, SE.; Nielsen, AM. *Fatty Alcohols: Anthropogenic and Natural Occurrence in the Environment*. The Royal Society of Chemistry; Cambridge, UK: 2008.
- Neidhardt FC, Bloch PL, Smith DF. Culture Medium for Enterobacteria. *J. Bacteriol.* 1974; 119:736–747. [PubMed: 4604283]
- Ranganathan S, Tee TW, Chowdhury A, Zomorodi AR, Yoon JM, Fu Y, Shanks JV, Maranas CD. An integrated computational and experimental study for overproducing fatty acids in *Escherichia coli*. *Metab. Eng.* 2012; 14:687–704. [PubMed: 23036703]
- Reiser S, Somerville C. Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. *J. Bacteriol.* 1997; 179:2969–2975. [PubMed: 9139916]

- Rowland O, Domergue F. Plant fatty acyl reductases: enzymes generating fatty alcohols for protective layers with potential for industrial applications. *Plant Science*. 2012; 193-194:28–38. [PubMed: 22794916]
- Rupilius W, Ahmad S. The Changing World of Oleochemicals. *Palm Oil Developments*. 2006; 44:15–28.
- Sampson EM, Bobik TA. Microcompartments for B12-dependent 1,2-propanediol degradation provide protection from DNA and cellular damage by a reactive metabolic intermediate. *J. Bacteriol.* 2008; 190:2966–71. [PubMed: 18296526]
- Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, Del Cardayre SB, Keasling JD. Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. *Nature*. 2010; 463:559–562. [PubMed: 20111002]
- Teerawanichpan P, Qiu X. Fatty acyl-CoA reductase and wax synthase from *Euglena gracilis* in the biosynthesis of medium-chain wax esters. *Lipids*. 2010; 45:263–273. [PubMed: 20195781]
- Tseng C, Albrecht J, Gunsalus RP. Effect of Microaerophilic Cell Growth Conditions on Expression of the Aerobic (cyoABCDE and cydAB) and Anaerobic Pathway Genes in *Escherichia coli*. *Microbiology*. 1996; 178:1094–1098.
- Voelker TA, Davies HM. Alteration of the specificity and regulation of Alteration of the Specificity and Regulation of Fatty Acid Synthesis of *Escherichia coli* by Expression of a Plant Medium-Chain Acyl-Acyl Carrier Protein Thioesterase. *J. Bacteriol.* 1994; 176(23):7320–7327. [PubMed: 7961504]
- Wang Q, Tappel RC, Zhu C, Nomura CT. Development of a new strategy for production of medium-chain-length polyhydroxyalkanoates by recombinant *Escherichia coli* via inexpensive non-fatty acid feedstocks. *Appl. Environ. Microbiol.* 2012; 78:519–27. [PubMed: 22101037]
- Willis RM, Wahlen BD, Seefeldt LC, Barney BM. Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. *Biochemistry*. 2011; 50:10550–8. [PubMed: 22035211]
- Xu P, Gu Q, Wang W, Wong L, Bower AGW, Collins CH, Koffas MAG. Modular optimization of multi-gene pathways for fatty acids production in *E. coli*. *Nat. Commun.* 2013; 4:1409. [PubMed: 23361000]
- Youngquist JT, Lennen RM, Ranatunga DR, Bothfeldt WH, Marner WD, Pfeleger BF. Kinetic modeling of free fatty acid production in *Escherichia coli* based on continuous cultivation of a plasmid free strain. *Biotechnol. Bioeng.* 2012; 109:1518–27. [PubMed: 22234725]
- Youngquist JT, Rose JP, Pfeleger BF. Free fatty acid production in *Escherichia coli* under phosphate-limited conditions. *Appl. Microbiol. Biotechnol.* 2013
- Yu D, Ellis HM, Lee E-C, Jenkins NA, Copeland NG, Court DL. An efficient recombination system for chromosome engineering in *Escherichia coli*. *Proc. Natl. Acad. Sci. U.S.A.* 2000; 97:5978–5983. [PubMed: 10811905]
- Yuan L, Dehesh K, Kridl J, Knauf V. Engineering plant thioesterases for altered substrate specificity. 1999:63.
- Zha W, Rubin-Pitel SB, Shao Z, Zhao H. Improving cellular malonyl-CoA level in *Escherichia coli* via metabolic engineering. *Metab. Eng.* 2009; 11:192–198. [PubMed: 19558964]
- Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD. Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. *Metab. Eng.* 2012; 14:653–60. [PubMed: 23026122]
- Zheng Y-N, Li L, Liu Q, Yang J, Wang X, Liu W, Xu X, Liu H, Zhao G, Xian M. Optimization of fatty alcohol biosynthesis pathway for selectively enhanced production of C12/14 and C16/18 fatty alcohols in engineered *Escherichia coli*. *Microb. Cell Fact.* 2012; 11:65. [PubMed: 22607313]

Highlights

- Metabolic engineering of *Escherichia coli* to produce 1-dodecanol and 1-tetradecanol
- Demonstrated tailoring of individual gene expression levels to improve production
- Highest reported yield (>0.13 g/g) of fatty alcohols on an unrelated carbon source
- Demonstrated constant production in prolonged stationary phase
- Successfully increased extracellular fraction of product by co-culturing with dodecane

Fig. 1. Fatty Alcohol Biosynthesis

a.) Schematic of metabolic pathways that lead to fatty alcohols. Fatty acid biosynthesis generates acyl-acyl-carrier proteins (acyl-ACP) that are the substrates for lipid synthesis, thioesterases (TE) and acyl-CoA reductases (ACR). The fatty aldehydes produced by ACR can be reduced to primary alcohols by aldehyde reductases (AR). Expression of ACR/AR pairs leads to the formation of fatty alcohols that match the predominant acyl-ACP species (i.e. 16 carbons in *E. coli*). Alternatively, medium chain length alcohols can be produced by using an acyl-ACP thioesterase to produce a smaller fatty acid. Free fatty acids are then converted to acyl-CoA thioesters, by acyl-CoA synthetases (AS) and subsequently reduced by ACR to aldehydes and by AR to alcohols. b.) Conversion of exogenously fed dodecanoic acid to 1-dodecanol by *E. coli* strains harboring *ptrc99a*-MAACR (MAACR contains both ACR and AR activities). To produce 1-dodecanol, β-oxidation must be blocked (Δ *fadE*) and ACS (FadD) activity must be increased from native levels. c.) Expression of MAACR also results in production of 1-hexadecanol in each strain. d.) Cultures of Δ *fadE* *pMAACR* supplemented with 100 mg/L hexadecanoic acid generated 76 mg/L of hexadecanol after 24 hours, whereas cultures of Δ *fadE* Δ *fadD* *pMAACR* generated 10-15 mg/L of hexadecanol, equivalent to unsupplemented Δ *fadE* *pMAACR* cultures.

Fig. 2. Comparison of Acyl-CoA Synthetases

E. coli MHS04 ($\Delta fadR \Delta fadD$) harboring one of four acyl-CoA synthetase expression plasmids (medium copy, P_{trc}) was fed dodecanoic acid. The rate of consumption was fastest for the strain expressing FadD. The control strain carried the empty pACYC plasmid. The error bars represent standard deviations from biological triplicate shake flask cultures.

Fig. 3. Comparison of Acyl-CoA Reductases

a.) The titer of 1-dodecanol produced by *E. coli* MHS01 ($\Delta fadE \Phi[P_{T_{rc}} fadD]$) when dodecanoic acid is exogenously supplied in the media. Each strain harbored one of three plasmids – pTRC99A, pTRC99A-ACR1, or pTRC99A-MAACR. The error bars represent standard deviations from biological triplicate shake flask cultures.

Fig. 4. Optimization of MAACR Expression

The combined titers of 1-dodecanol and 1-tetradecanol as well as residual dodecanoic and tetradecanoic acid were measured for a.) *E. coli* MHS03 ($\Delta fadE::trcBTE \Phi(P_{Trc-fadD})$) and b.) different copy number vectors. The Empty plasmid was ptrc99a. c.) The plasmid copy number determined by qPCR (relative to *ompA*) increases from left to right. Plasmids conferring resistance to ampicillin were present in fewer copies after 24 hours likely due to the loss of ampicillin over time. In all but the lowest copy number plasmid, high titers of FFA were observed. The highest alcohol titers were achieved when MAACR was expressed on a low copy vector, independent of the number of copies of BTE. Error bars represent standard deviation of biological triplicate shake flask cultures.

Fig. 5. Optimization of FadD Expression

a.) Dodecanol and b.) tetradecanol production as a function of the relative expression level of acyl-CoA synthetase (*fadD*) in *E. coli* strains harboring pBTRK-MAACR compared to native expression (TY31). Error bars represent standard deviation from biological triplicate shake flask cultures.

Fig. 6. Production of Fatty Alcohols in Co-Culture

a.) Final observed fatty alcohol titer breakdown in strains MHS03 (1 copy of BTE), TY30 (2 copies of BTE), and TY34 (3 copies of BTE) harboring pBTRK-MAACR after being run in a stirred bioreactor. OL refers to the presence of a dodecane overlayer added during fermentations b.) The titer of fatty alcohol produced from *E. coli* TY34 pBTRK-MAACR fed-batch cultivations is plotted as a function of time. c.) The relative quantity of metabolic products, as percentage of fed carbon, shows large percentages going to CO₂, acetate, biomass, and fatty alcohols. d.) The off-gas [CO₂] suggests that a metabolic steady state is achieved ~30 h post induction. e.) Co-culturing *E. coli* with dodecane increases the amount of fatty alcohol found outside the cell. f.) Glucose consumption was nearly linear over the fed-batch portion of the culture.

Table 1

Strains and plasmids used in this study

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
Strains		
<i>E. coli</i> K-12	F ⁻ λ <i>ilvG</i> ⁻ <i>rfb</i> -50 <i>rph</i> -1	ECGSC
MG1655		
<i>E. coli</i> DH10B	F ⁻ <i>mcrA</i> Δ (<i>mrr</i> - <i>hsdRMS</i> - <i>mcrBC</i>) Φ 80 <i>lacZ</i> Δ M15 Δ <i>lacX</i> 74 <i>recA1</i> <i>endA1</i> <i>araD</i> 139 Δ (<i>ara</i> , <i>leu</i>)7697 <i>galU</i> <i>galK</i> λ <i>rpsL</i> <i>nupG</i>	Invitrogen
<i>E. coli</i> DH5 α	F ⁻ Φ 80 <i>lacZ</i> Δ M15 Δ (<i>acZYA</i> - <i>argF</i>) U169 <i>recA1</i> <i>endA1</i> <i>hsdR17</i> (<i>r</i> _K ⁻ , <i>m</i> _K ⁺) <i>phoA</i> <i>supE</i> 44 λ <i>thi</i> -1 <i>gyrA</i> 96 <i>relA1</i>	Invitrogen
<i>E. coli</i> DY330	F ⁻ λ <i>rph</i> -1 INV(<i>rrnD</i> , <i>rrnE</i>) Δ <i>lacU</i> 169 <i>gal</i> 490 <i>pgl</i> Δ 8 λ cI857 Δ (<i>cro</i> - <i>bioA</i>)	(Yu et al., 2000)
<i>Pseudomonas putida</i> KT2440	Source for PP_0763	ATCC 47054 TM
MHS01	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> Φ (P _{T_{trc}} - <i>fadD</i>)	This work
MHS02	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> ::trcBTE	This work
MHS03	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> ::trcBTE Φ (P _{T_{trc}} - <i>fadD</i>)	This work
MHS04	MG1655 Δ <i>araBAD</i> Δ <i>fadR</i> Δ <i>fadD</i>	This work
DE	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> Δ <i>fadD</i>	This work
E	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i>	(Agnew et al., 2012)
RL08	MG1655 Δ <i>araBAD</i> Δ <i>fadD</i>	(Lennen et al., 2010)
TY19	MG1655 Δ <i>araBAD</i> Δ <i>fadR</i> Δ <i>fadE</i> ::trcBTE	This work
TY27	MG1655 Δ <i>araBAD</i> Δ <i>fadD</i> Δ <i>fadE</i> ::trcBTE	This work
TY30	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> ::trcBTE Δ <i>fadAB</i> ::trcBTE Φ (P _{T_{trc}} - <i>fadD</i>)	This work
TY31	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> ::trcBTE Δ <i>fadAB</i> ::trcBTE	This work
TY32	MG1655 Δ <i>araBAD</i> Δ <i>fadR</i> Δ <i>fadE</i> ::trcBTE Δ <i>fadAB</i> ::trcBTE	This work
TY33	MG1655 Δ <i>araBAD</i> Δ <i>fadD</i> Δ <i>fadE</i> ::trcBTE Δ <i>fadAB</i> ::trcBTE	This work
TY34	MG1655 Δ <i>araBAD</i> Δ <i>fadE</i> ::trcBTE Δ <i>fadAB</i> ::trcBTE Δ <i>ackApta</i> ::trcBTE Φ (P _{T_{trc}} - <i>fadD</i>)	This work
Plasmids		
pBTRKtrc	P _{trc} promoter, pBBR1 origin, Kan ^R	This work
pUCtrc	P _{trc} promoter, pUC origin, Amp ^R	This work
pACYCtrc	P _{trc} promoter, pACYC origin, Cm ^R	This work
pACYC-fadD	pACYCtrc carrying <i>fadD</i> under P _{trc} control, Cm ^R	This work
pACYC-PP0763	pACYCtrc carrying PP_0763 (<i>P. putida</i>) under P _{trc} control, Cm ^R	This work
pACYC-fadD6	pACYCtrc carrying <i>fadD6</i> (<i>M. tuberculosis</i>) under P _{trc} control, Cm ^R	This work
pTrc99A	P _{Trc} promoter, pBR322 origin, Amp ^R	(Amann et al., 1988)
ACR1	pTrc99A carrying <i>acr1</i> from <i>Acinetobacter calCoAceticus</i> under P _{trc} control, Amp ^R	This work
FAR6	pTrc99A carrying <i>far6</i> from <i>Arabidopsis thaliana</i> under P _{trc} control, Amp ^R	This work
ptrc99a-MAACR	pTrc99A carrying MAACR from <i>Marinobacter aquaeolei</i> under P _{trc} control and fused to a maltose binding protein, Amp ^R	This work
pBTRK-MAACR	pBTRKtrc containing MAACR	This work

Strain/Plasmid	Relevant Genotype/Property	Source or Reference
pACYC-MAACR	pACYCtrc containing MAACR	This work
pUCtrc-MAACR	pUCtrc containing MAACR	This work

Table 2

Oligonucleotide primers used in this study

Primer Name	Sequence (5' to 3')
1. Forward rnb	gaaaggttttcaccattcgatgggtCggtgcctaagtagtgagctaac
2. Reverse before lacI	gaaaggttttcaccattcgatgggtCggtgcctaagtagtgagctaac
3. Forward before lacI	atcgaatgggtgcaaacctttc
4. Reverse from rnb to get MCS, ptrc, and lacI	gaaacgcaaaaaggccatcc
5. Gibson MAACR fwd (MAACR gib F)	acacaggaaacagaccatCACCAACAAGGACCATAGC
6. Gibson MAACR rev (MAACR gib R)	tcacccgcaaaacagcTTATCAGTGATGGTGATGATGG
7. fadD fwd	gaaaagagctcgggtaccAGGAGGTATAAGAAttgaagaaggtttggcttaacc
8. fadD rev	gaaaagtcgactctagattaTCAGGCTTTATTGTCCACTTTGC
9. BTEack-pta_int_F	atgttaatacataaatgtcgggtgcatcatgcgctacgctcGGCATGCGTTCCTAT TCCGAAGTTCC
10. BTEack-pta_int_R	agcgcaaaagctgcggatgatgacgagattactgctgctgTACATCCGCCAAA ACAGCCAAG
11. PP0763 fwd	GAGAAAgagctcgggtaccAGGAGGTAAAATAATGTTGCAGAC ACGCATCATC
12. PP0763 rev	GAAAAGcctgcaggtctagaTTAGTGATGGTGATGGTGATGCA ACGTGGAAAGGAACGC
13. rev from start of MCS (ptrc gib R)	atggtctgtttcctgtgtg
14. fwd from end of MCS (ptrc gib F)	gctgttttggcggtgag
15. MAACR qPCR fwd	ctatgtctcctcgaaatc
16. MAACR qPCR rev	gaatcgtagatcttgggtg
17. ompA qPCR fwd	tgttgagtacgcgacactc
18. ompA qPCR rev	gttggtccgacgagtg