Production of 1-octanol in *Escherichia coli* by a high flux thioesterase route

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Abstract

1-octanol is a valuable molecule in the chemical industry, where it is used as a plasticizer, as a precursor in the production of linear low-density polyethylene (LLDPE), and as a growth inhibitor of tobacco plant suckers. Due to the low availability of eight-carbon acyl chains in natural lipid feedstocks and the selectivity challenges in petrochemical routes to medium-chain fatty alcohols,1-octanol sells for the highest price among the fatty alcohol products. As an alternative, metabolic engineers have pursued sustainable 1-octanol production via engineered microbes. Here, we report demonstration of gram per liter titers in the model bacterium *Escherichia coli* via the development of a pathway composed of a thioesterase, an acyl-CoA synthetase, and an acyl-CoA reductase. In addition, the impact of deleting fermentative pathways was explored *E. coli* K12 MG1655 strain for production of octanoic acid, a key octanol precursor. In order to overcome metabolic flux barriers, bioprospecting experiments were performed to identify acyl-CoA synthetases with high activity towards octanoic acid and acyl-CoA reductases with high activity to produce 1-octanol from octanoyl-CoA. Titration of expression of key pathway enzymes was performed and a strain with the full pathway integrated on the chromosome was created. The final strain produced 1-octanol at 1.3 g/L titer and a >90% C_8 specificity from glycerol. In addition to the metabolic engineering efforts, this work addressed some of the technical challenges that arise when quantifying 1-octanol produced from cultures grown under fully aerobic conditions where evaporation and stripping are prevalent.

Keywords:

Octanol; octanoic acid; thioesterase; Escherichia coli; oleochemical; metabolic engineering

Introduction

1-octanol is an aliphatic molecule widely used in the chemical industry as a plasticizer, as a precursor to surfactants and fatty amines, as a plant sucker growth inhibitor in the tobacco industry (USDA,A.A.D. for the U.N.O.P., 2016), as a biodiesel additive (Akhtar et al., 2015), and as an intermediate to make a co-monomer (via dehydration to 1-octene) (Van Leeuwen, et al., 2011) for the production of linear low-density polyethylene (LLDPE). Octanol often sells for the highest price (~\$1.5/lb) among fatty alcohols (C_{12} and C_{16} chain alcohols sell for ~\$0.8/lb) (Pfleger et al., 2015), in part because octanoic acid and other eight-carbon oleochemicals are not abundant in common lipid sources (Yan and Pfleger, 2020). Instead, 1-octanol is produced alongside other linear alcohols via iterative oligomerization chemistries from smaller petrochemicals. However, , the Ziegler process produces an alcohol stream containing only ~17% octanol when fed ethylene, hydrogen, and aluminum (Falbe et al., 2011). A proportional quantity of aluminum hydroxide is also produced in the process thereby limiting its scale. Thus, the lack of a sustainable, low-cost supply motivates a search for alternative routes of production.

Biological conversion of sugars and other feedstocks to 1-octanol is potentially feasible at current sugar prices if high yields are achieved (Pfleger et al., 2015). However, unlike other products (i.e. short-chain alcohols, short-chain acids, amino acids, among others) which are advanced in their development, production of 1-octanol remains in the proof-of-concept stage (Akhtar et al., 2015; Sattayawat et al., 2020). Microbial synthesis of oleochemicals has advanced substantially in the last decade (Yan and Pfleger, 2020), yet the Ziegler process remains the dominant technology for the industrial production of medium-chain oleochemicals. Chain-length selectivity remains a challenge because highly active enzymes that selectively target C_8-C_{12} intermediates in fatty acid biosynthesis and β-oxidation are not common in nature (Dellomonaco et al., 2011; Mehrer et al., 2018). In addition, medium-chain products are inherently more toxic to the cell than more common long-chain oleochemicals because they disrupt the structure and function of microbial lipid membranes. Addressing these issues are two of the main focus points in current efforts to engineer medium-chain oleochemical production (Sarria et al., 2017).

1-octanol can be made biologically from the reduction 1-octanal via an aldehyde reductase (**Figure 1**). In a cellular context, fatty aldehydes are often a by-product of reactive oxygen species and long-chain phospholipids. Fatty aldehydes have a propensity to form adducts with amino groups in proteins and DNA causing cellular damage. To counteract this, cells have evolved very active aldehyde reductases (Adh) that protect them from these adduct-forming reactions (Perez et al., 2008). Therefore, in producing 1-octanol from 1-octanal the major concern is how to synthesize the latter with high titer and chain-length specificity. As can be seen in **Figure 1**, 1-octanal can be made enzymatically from three metabolic precursors: octanoyl-ACP (Acyl-Carrier Protein), octanoyl-CoA (Coenzyme A), and octanoic acid. These metabolites are derived either from fatty acid biosynthesis (FAB) or via thiolase-driven pathways such as the reversal of the β-oxidation (rβOX) (Dellomonaco et al., 2011). Three different pathways have been demonstrated for the production of octanol from octanoyl-ACP. In the simplest approach, acyl-ACPs can be directly reduced using an acyl-ACP reductase (AAR) (Liu et al., 2014). In a second approach, octanoyl-ACP can be hydrolyzed to octanoic acid via a C_8-ACP thioesterase (TE), followed by direct reduction with a carboxylic acid reductase (CAR) (Akhtar et al., 2015). Alternatively, after free octanoic acid is produced from octanoyl-ACP, the acyl moiety can be activated to octanoyl-CoA using an acyl-CoA synthetase (ACS) and subsequently reduced with an acyl-CoA reductase (ACR) (Youngquist et al., 2013). The enzymes, regulation, and engineering of FAB and rβOX pathways have been thoroughly reviewed elsewhere (Cronan, 2003; d'Espaux et al., 2015; Lennen and Pfleger, 2013, 2012; Marella et al., 2018; Pfleger et al., 2015; Sarria et al., 2017; Yan and Pfleger, 2020). The highest yielding routes would use AAR or ACR to directly convert intermediates in the FAB and rβOX pathways to octanal. Unfortunately, enzymes capable of selectively performing these reactions at high rates have yet to be discovered or engineered. Therefore, pools of eight carbon molecules must be created and allowed to accumulate to be converted by downstream reductive enzymes. Thioesterases, particularly those from plants, have been shown to be highly selective towards specific chain-lengths. When β-oxidation is disrupted, chain-length selective acyl-ACP thioesterases can be used to create pools of desired fatty acids or acyl-CoAs with reactivation by acyl-CoA synthetases. Terminal reductions can then be performed by substrate promiscuous CAR or ACR enzymes. In this strategy, the thioesterase dictates selectivity and the lack of other acyl-CoA metabolism enables substrates to build to levels where CAR/ACR activity is substantial.

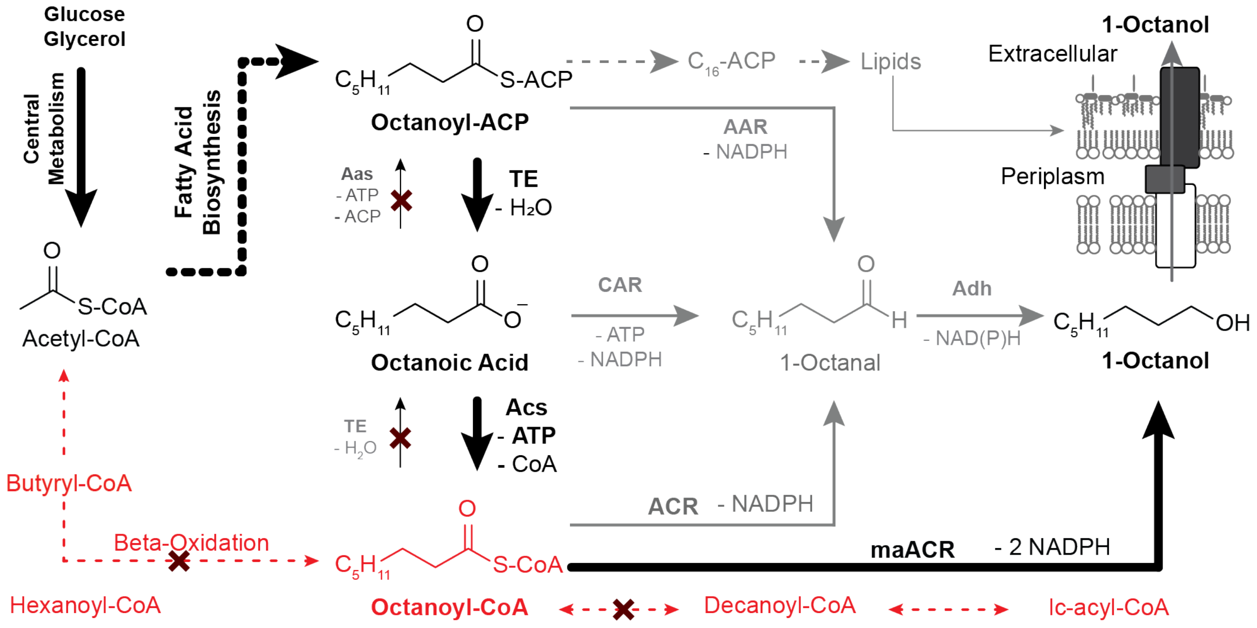

Figure 1. Oleochemical Metabolism and Routes to 1-Octanol.

Oleochemicals are derived from acetyl-CoA that is generated in central metabolism from exogenous feedstocks (e.g. glucose, glycerol). Longer acyl-chains are synthesized via fatty acid biosynthesis (black hashed lines) or beta-oxidation (red hashed lines). The strategy applied in this project uses a heterologous thioesterase with activity towards octanoyl-ACP to divert flux from fatty acid biosynthesis to octanoic acid. Octanoic acid is then activated to octanoyl CoA by an acyl-CoA synthase (ACS). Beta oxidation is blocked such that a pool of octanoyl-CoA accumulates. Octanoyl-CoA is reduced twice by a dual-function acyl-CoA reductase (ACR) and aldehyde reductase (Adh) encoded by the *Marinobacter aquaeolei* ^{Ma}ACR. Alternative routes (in grey), including 1.) CAR mediated reduction of octanoic acid, 2.) direct reduction of octanoyl-ACP to octanal, and 3.) reduction of octanoyl-CoA to octanal by a distinct ACR were not pursued.

In previous work, we engineered an acyl-ACP thioesterase (CpFatB1) from the shrub *Cuphea palustris* to increase its activity while maintaining its ~90% specificity for the C8 acyl-chains (Hernandez-Lozada et al., 2018). The improved variant was isolated via a genetic selection from an error-prone library of the CpFatB1 thioesterase. The selection strain, *E. coli* MG1655 *ΔlipB*, lacked the ability to synthesize a lipoylated E2 domain of pyruvate dehydrogenase from octanoyl-ACP. The deletion forces the *ΔlipB* strain to use a salvage pathway, through LplA and LipA enzymes, that relies on exogenous octanoic or lipoic acids. Without these compounds present in the media, cells cannot grow in aerobic conditions (Zhao et al., 2003). Active thioesterases provided a route to produce the required octanoic acid. The best variant isolated from the selection, CpFatB1.2-M4-287, which hereafter will be referred as CpFatB1*, demonstrated a 15-fold improvement in k_{cat}. The strong activity allowed us to express a single chromosomal copy of CpFatB1* in the *fadD* loci and produce octanoic acid at titers up to 1.7 g/L in rich media. This level of performance motivated development of the downstream components of the pathway to produce octanol and other 8-carbon oleochemical products. Recently, Jones and colleagues demonstrated the CAR-mediated route to produce octanol using our improved thioesterase (Sattayawat et al., 2020).

In this manuscript, we explored the variables that lead to rapid conversion of octanoic acid to octanol via the ACS/ACR route and the genetic and environmental changes that lead to high flux to octanol from sugars. We first explored fermentation variables including host strain, fermentative pathway knockouts, and growth media to maximize production of octanoic acid, the key metabolic intermediate, from sugars. Next, we tested a series of acyl-CoA synthetases (ACS) to replace *E. coli* FadD which has been shown to have very low activity on octanoic acid (Iram & Cronan, 2006). In addition, we characterized the pattern of evaporation of 1-octanol in a shake flask under our fermentation conditions and found that dodecane can be used to alleviate evaporation issues. We then screened acyl-CoA reductases (ACR) in order to identify variants with high catalytic activity towards octanoyl-CoA under aerobic conditions. Finally, we assembled the full pathway to demonstrate 1.3 g/L 1-octanol production from sugars in shake flasks from a strain with the 1-octanol pathway fully incorporated in the chromosome. These results represent a 2.3-fold improvement to the best result in the literature and the first demonstration of 1-octanol production at gram per liter titers in a plasmid-free strain.

Materials and Methods

Chemicals, Strains and Plasmids

Chemicals were purchased from Sigma Aldrich (St. Louis, MO) and Fisher Scientific (Waltham, MA). Oligonucleotides and gene fragments were purchased from Integrated DNA Technologies (Coralville, IA). Enzymes were purchased from New England Biolabs (Ipswich, MA). DNA purification kits were purchased from Qiagen (Venlo, Netherlands). All cultures were started from single colonies grown on LB agar isolated from freezer stocks stored in 15% glycerol. Overnight cultures of strains were grown in LB media at 37°C in a rotary shaker at 250 r.p.m. In experiments where plasmids were used, media was supplemented with the appropriate antibiotics (carbenicillin, 100 μg/mL; kanamycin, 50 μg/mL; chloramphenicol, 34 μg/mL). See **Table S1** for a detailed list of strains and **Table S2** for a list of plasmids used in this study. Annotated sequence maps are provided for all plasmids as supplementary files.

Chromosomal integrations

Chromosome modifications (deletions, insertions, replacements) were performed with a two plasmid system, as described previously (Mehrer et al., 2018). Briefly, a 50 mL cell culture containing the pMP11 vector was inoculated with 1 mL of dense overnight culture, grown at 30 °C for an hour, and induced with 1% (w/v) arabinose to express the λ Red genes. The culture was grown to mid-exponential phase (OD_{600} = 0.6) and prepared for electroporation of the double-stranded DNA repair template and the guide RNA corresponding to the chromosomal target locus. After electroporation, the transformants were recovered with SOC media, grown at 30 °C for 3 hours, and plated on antibiotic selection plates. Integrants were screened by colony PCR and verified with Sanger sequencing. The gRNA and pMP11 plasmids were subsequently cured from the successful integrant. DNA repair templates were cloned using the BW25141/pKD13 system and were designed with 500 base pairs of homology upstream and downstream of the integration site (Datsenko et al., 2000, Haldimann et al., 2001). Guide RNAs were designed with the Atum designer tool (atum.bio).

Free fatty acid experiments

For experiments designed to produce free fatty acids (**Figure S1**), single colonies were grown overnight in LB media supplemented with the appropriate antibiotics. For minimal media experiments (**Figure S1F**), 50 µL of cultures grown overnight in LB media was used to inoculate 5 mL minimal media seed cultures that were ultimately used to inoculate experimental cultures. Seed cultures from three independent colonies were used to inoculate 50 mL of media in a 250 mL shake flask at an initial OD_{600} = 0.05. Cells were allowed to grow at 37 °C to OD_{600} = 0.2 before induction with IPTG; at which point cultures were incubated with shaking at 30 °C for 24 h. IPTG concentrations used were as follows: for **Figure S1A-C** induction is indicated on X axis; for **Figure S1D-F,** 50 µM IPTG was used which corresponds to the optimal induction level for the pTRC99a-CpFatB1* plasmid (see **Figure S1C**); for **Figures 2 and 3,** 1 mM IPTG was used. Media used for **Figure S1A-D** was MOPS rich media supplemented with glycerol as described elsewhere (Kim et al., 2015), but with the following changes: 1.39 mM Na_2HPO_4, no biotin, thiamine or sodium selenite added). For **Figure S1E**, we replaced the glycerol from the MOPS rich media described above with glucose. For **Figure S1F** we used MOPS minimal media phosphate limited as described elsewhere (Youngquist et al., 2013). Experiments in which octanoic acid was supplied externally (**Figures 2 and 3**) were done in LB media.

Fatty acid extraction, methylation and quantification

Fatty acid samples were prepared by collecting the equivalent of 2.5 mL culture in a 10 mL glass centrifuge tube, such that 2.5 mL was collected when no dodecane overlay was added to the culture, 2.75 mL when 1:10 dodecane to media ratio was used, and 3.0 mL when 1:5 dodecane to media was used. 50 μL of internal standard containing 12.5 mg/mL nonanoic acid and 1.25 mg/mL pentadecanoic acid was added to the samples. The extraction and methylation procedure was performed as described in previous work (Grisewood et al., 2017). For GC quantification, samples were analyzed using a GC-FID model Shimadzu GC-2010 equipped with an AOC-20i auto-injector and a 30 m, 0.25 mm ID RTX-5 column. The column oven temperature protocol was 100°C for 2 min, ramp to 125°C (at 7°C/min), ramp to 235°C (at 10°C/min), fast ramp to 250°C, and hold for 2 min. All error bars represent the standard error of three biological replicates.

Fatty alcohol experiments

For experiments designed to produce fatty alcohols (**Figure 3B, Figure 4**) single colonies were grown overnight in LB media supplemented with the appropriate antibiotics for maintaining plasmids. Overnight cultures from three single colonies were used to inoculate three 250 mL shake flasks containing 50 mL of media (for **Figure 3B** we used LB + 500 mg/L octanoic acid; for **Figure 4** MOPS rich media glycerol described elsewhere (Kim et al., 2015) with the following changes: 1.39 mM Na_2HPO_4, no biotin, thiamine or sodium selenite added) at an initial OD_{600} = 0.05. Cells were allowed to grow at 37 °C to OD_{600} = 0.2 before inducing with 1mM IPTG (**Figure 3B, Figure 4, last two panels)** or 50 µM IPTG (**Figure 4, first three panels**), 10 mL dodecane was added to control evaporation and the cultures were moved to 30 °C for up to 24 h after induction. For octanoic acid consumption experiments in the presence of ACS and ACR (**Figure S4**) 100 µL of a 250 mg/mL octanoic acid solution in ethanol was added immediately after induction.

Fatty alcohol extraction and quantification

Before collecting fatty alcohol samples, 100 μL of an internal standard containing 125 mg/mL nonanol, and 12.5 mg/mL pentadecanol was added to the shake flasks and allowed to shake for 5 min to ensure mixing. Cultures in the presence of dodecane were constantly mixed right before the sample was taken. Fatty alcohol samples were prepared by collecting the equivalent of 2.5 mL culture (same as free fatty acid extraction section) into a 10 mL glass centrifuge tube. Samples were then extracted into 1 mL hexane by vortexing the solution for 3 min at max speed on a Vortex Genie 2 purchased from Scientific Industries (Bohemia, NY). Samples were centrifuged at 3000 x g for 10 min and the hexane layer was injected in a GC-FID. Samples were analyzed using a GC-FID model Shimadzu GC-2010 equipped with an AOC-20i auto-injector and a 30 m, 0.25 mm ID RTX-5 column. The column oven temperature protocol was 100°C for 2 min, ramp to 125°C (at 7°C/min), ramp to 235°C (at 10°C/min), fast ramp to 250°C, and hold for 2 min. All error bars represent the standard error of three biological replicates.

Results and Discussion

Optimizing Thioesterase Expression

In *E. coli*, long-chain acyl-ACPs are the key regulatory signal that controls flux through fatty acid biosynthesis. Therefore, we hypothesized that expression of the thioesterase CpFatB1*, which creates a competing metabolic sink and thereby depletes the abundance of long-chain acyl-ACPs, was a key step to optimize production of 1-octanol. In prior work, a MG1655 *ΔfadD* strain produced the most octanoic acid when expressing CpFatB1* at sub-saturating induction conditions (Hernandez-Lozada et al., 2018). Therefore, we explored thioesterase expression from the common K12 MG1655 and LS5218, an *E. coli* K12 strain obtained after selection on decanoate and then butyrate (Rand et al., 2017). LS5218 was a superior host for producing fatty alcohols under anaerobic conditions (Mehrer et al., 2018) and is widely used for producing poly(3-hydroxyalkanoates) (Agnew et al., 2012) under aerobic conditions.

We tested expression of CpFatB1* in these two strains using three vectors, 1.) integration of a P_{TRC}-CpFatB1*expression cassette (strong ribosome binding site, RBS) in the *fadD* loci of each strain, 2.) expression of CpFatB1* with a strong RBS on low-copy pBTRK plasmids, and 3.) expression of CpFatB1* with a weak RBS on high-copy pTRC99A plasmids. Cells were inoculated from overnight cultures into 50 mL of rich glycerol media, induced at OD_{600} of 0.2 with 0 μM, 20 μM, 50 μM and 1000 μM IPTG and incubated with shaking at 30 °C for 24 h after induction. The titer of the plasmid-free strains both peaked at maximum induction with the MG1655 strain (1.7 g/L titer octanoic acid) outperforming the LS5218 strain (0.79 g/L) when maximally induced (**Figure S1A**). In prior studies, we have observed reduced titers and cell fitness as thioesterase expression was maximized (Lennen et al., 2010; Hoover et al., 2011;

Youngquist et al., 2013). The lack of a maximum in octanoic acid titer suggested that further increases could be achieved with additional CpFatB1* expression. Therefore, we tested CpFatB1* expression from plasmids. When low copy plasmids were used but the RBS strength was held constant (**Figure S1B**), the titer from MG1655 cultures peaked at 1.5 g/L octanoic acid when uninduced while the titer of LS5218 cultures peaked when induced at 50 µM IPTG with 0.94 g/L. Finally, we lowered the RBS strength and moved the genes to a high copy plasmid, the MG1655 titer peaked when induced with 50 µM IPTG with 1.5 g/L whereas LS5218 titer peaked at 1 mM IPTG with 1.1 g/L (**Figure S1C**). These trends suggested that leaky expression from the low-copy plasmid was sufficient to produce the maximum amount of octanoic acid in MG1655 and further expression led to diminishing titers, analogous to prior studies. The preceding data shows that for all expression systems *E. coli* MG1655 outperformed LS5218 in terms of octanoic acid titer. Therefore, we abandoned further development of LS5218 for 1-octanol production and performed all further experiments in K12 MG1655.

Blocking Alternative Carbon Sinks

When rapidly growing, *E. coli* is known to produce overflow metabolites, including acetate and lactate, that can reduce the final titer of desired products (Atsumi et al., 2008). In prior studies, we observed up to 7% of carbon flux being directed to acetate instead of the desired dodecanoic acid (Youngquist et al., 2012). Therefore, we created derivatives of MG1655 Δ*fadD* with additional gene deletions to block acetate and lactate secretion: phosphate acetyltransferase (*pta*), pyruvate oxidase (*poxB*), and lactate dehydrogenase (*ldhA*). These strains were transformed with pTRC99a-CpFatB1*. Cultures of each strain were grown in three media conditions, rich media with glycerol, rich media with glucose, and phosphate-limited minimal media with glucose. Cells were induced with 50 µM IPTG (optimal level from **Figure S1C**), and incubated with shaking at 30°C for 24 h after induction. In rich media, the deletion strains generated less octanoic acid than the base strain containing all fermentation pathways (**Figure S1D-E**). In phosphate-limited MOPS minimal media supplemented with glucose, all strains produced 0.40-0.49 g/L and no increase in titers was seen for having the additional deleted pathways (**Figure S1F**). Overall, we did not find that fermentative pathways were a major carbon sink when grown in batch cultures.

Acyl-CoA Synthetase

The next enzymatic step towards production of octanol is activation of octanoic acid via an acyl-CoA synthetase (ACS). We tested ACS homologs, *Pseudomonas putida* Pp-0763 (Wang et al., 2012; Agnew et al., 2012) and *Mycobacterium tuberculosis* ^{Mt}FadD6 (Arora et al. 2005) to evaluate their activity towards octanoic acid *in-vivo* relative to that of *E. coli* ^{Ec}FadD. These ACSs have been shown in the past to have wide substrate specificity, including medium-chain activity. In addition, ^{Pp}0763 was successfully used to synthesize C_8 and C_{10} polyhydroxyalkanoates (Wang et al., 2012). In the past, we have tested these enzymes for dodecanoic acid activity but were unable to outperform ^{Ec}FadD (Youngquist et al., 2013). However, ^{Ec}FadD has particularly weak enzymatic activity towards acyl-chains of less than ten carbons and efforts to alter its substrate preference have been met with limited success (Iram and Cronan, 2006; Ford et al., 2015).

In this experiment, we expressed each ACS candidate from a plasmid in strain MHS04 (*E. coli* MG1655 Δ*fadR* Δ*fadD*) and cultured cells in the presence of octanoic acid (Youngquist et al., 2013). The Δ*fadR* mutation in MHS04 deregulates expression of enzymes in the β-oxidation pathway (**Figure 2A**) and the Δ*fadD* deletion makes plasmid-based ACS expression necessary for activation of octanoic acid to octanoyl-CoA. Cells were inoculated from LB media overnight cultures into 50 mL of LB media with 250 mg/L octanoic acid, induced at OD_{600} 0.2 with 1 mM IPTG and grown at 30°C with shaking for an additional 12 h post induction. Cells carrying ^{Mt}FadD6 were able to consume most of the octanoic acid by 5 h post induction, and cells carrying ^{Pp}00763 were able to consume most of the octanoic acid by 8 h (**Figure 2B**). Cells expressing ^{Ec}FadD lagged behind and were unable to consume the octanoic acid in 12 hours. This data suggests that both ^{Mt}FadD6 and ^{Pp}00763 are active on octanoic acid and are suitable enzymes for assembling the pathway toward 1-octanol. For the rest of this study, we used ^{Mt}-FadD6 when expressing an acyl-CoA synthetase.

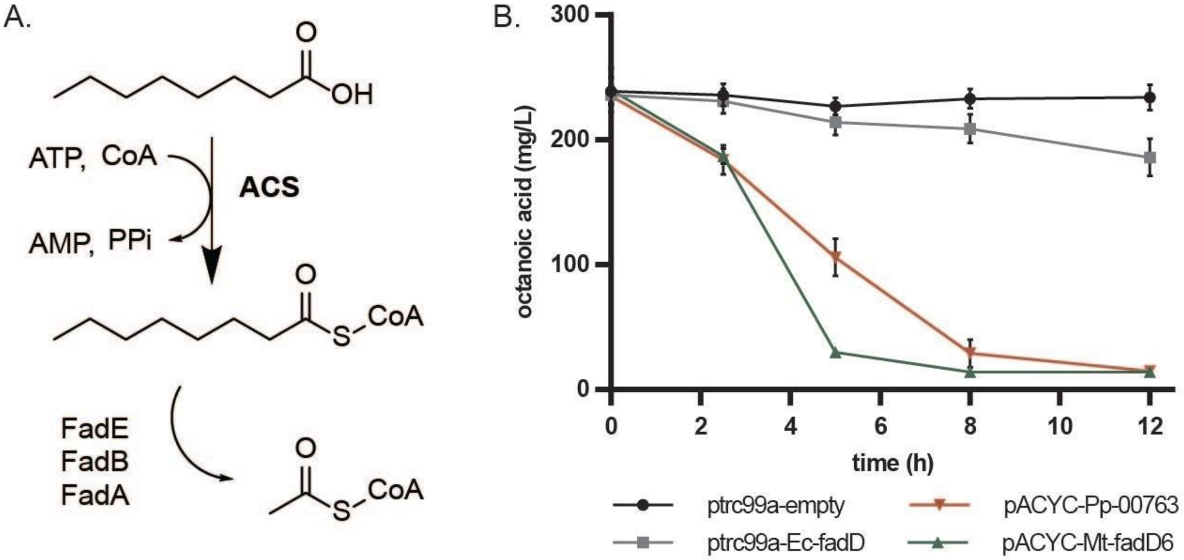

Figure 2. Acyl-CoA Synthetase with activity on octanoic acid.

(A) *E. coli* MHS04 (MG1655 Δ*fadR* Δ*fadD*) cells carrying plasmids with various acyl-CoA synthases are tested for their ability to convert octanoic acid to acetyl-CoA via deregulated β-oxidation. (B) MHS04 carrying ACS plasmids were grown in LB media with 250 mg/L octanoic acid (see materials and methods), and the octanoic acid consumption was tracked over time. ^{Mt}FadD6 was the best performer with most of the octanoic acid being consumed after 5 hours.

1-Octanol evaporation

We measured the evaporative loss of octanol from similar environments to assess if accurate titering was possible. In preliminary studies, we detected 1-octanol odors in production experiments, however, in samples taken after 24 h of incubation, a 1-octanol peak was not detected by gas chromatography. We set up shake flasks containing 50 mL of LB media supplemented with 250 mg/L of 1-octanol, incubated the flasks at 37°C with shaking, and took samples at different times. After 12 h, 85% of the 1-octanol had been lost from the system, presumably to evaporation or air stripping from the LB media (**Figure S2**). Prior studies used dodecane in cell cultures as a distinct organic phase to provide a product sink (Youngquist et al., 2013). We explored the effect of dodecane on the loss of octanol from flasks. We repeated the experiment by adding 5 mL (1:10) and 10 mL (1:5) dodecane to the 50 mL LB media supplemented with 250 mg/L 1-octanol and tracked the evaporation of 1-octanol over time. Interestingly, addition of dodecane reduced octanol loss but led to underestimation of octanol titers by approximately 20-25%. The underestimation stems in part from challenges associated with sampling an accurate volume of both phases, something we corrected using an internal standard in the production flask (**Figure S3**). Based on these results, all 1-octanol quantification was performed using cultures containing 1:5 v:v ratio of dodecane:media.

Acyl-CoA reductase

We next sought an ACR that would allow us to convert the octanoyl-CoA generated from the activity of CpFatB1* and ACS to 1-octanol. In an initial test, we showed that both ACS and ACR heterologous activities were required for consumption of exogenous octanoic acid in strain DE (*E. coli K12* MG1655 *ΔfadD ΔfadE*) (**Figure S4**). This test used the previously characterized ACR from *Marinobacter aquaeolei* (Willis et al., 2011) that our group used to produce dodecanol (Youngquist et al., 2013). In search of more effective enzymes, we tested eight additional acyl-CoA reductases identified in prior work (Mehrer et al., 2018) using the Enzyme Similarity Tool (Gerlt et al., 2015) and tested in a strain engineered to produce medium-chain fatty alcohols as anaerobic fermentation products. In order to test these variants, we fed octanoic acid to cultures of DE harboring ACS plasmid pACYC-^{Mt}fadD6 and various ACR variants on pTRCK plasmid while monitoring 1-octanol production over time (**Figure 3B**). In addition to ^{Ma}ACR, we found that ^{Mt}ACR from *Methylibium sp.,* and ^{Mb}ACR from *Marinobacter BSs20148* were able to have high turnover of octanoyl-CoA to 1-octanol. In the work by Mehrer and coworkers, ^{Mt}ACR was seen to express well and produce alcohols in anaerobic conditions, but ^{Mb}ACR did not express or produce alcohols. Interestingly, we found that both of them produce alcohols at the same levels as ^{Ma}ACR in aerobic conditions. It is not clear which of these enzymes produced the most 1-octanol from aerobic conditions since there is some evaporation occurring simultaneously. For this reason, we decided to continue using ^{Ma}ACR throughout the study. Furthermore, after 24 h the levels of 1-octanol accumulated was 30-50% percent lower than the 18 h time point suggesting that there is still considerable evaporation occurring even in the presence of dodecane and the actual 1-octanol production might be higher than the accumulation seen in the flask.

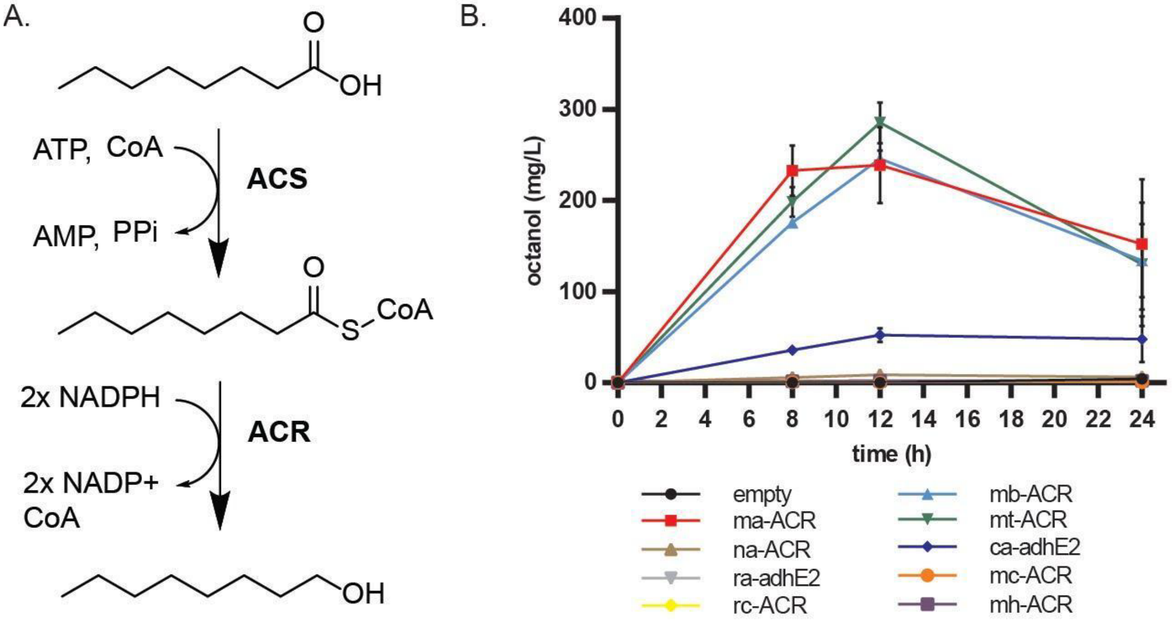

Figure 3. Production of 1-octanol from octanoic acid.

(A) *E. coli* DE (MG1655 Δ*fadD* Δ*fadE*) cells carrying plasmids with ^{Mt}FadD6 ACS and various acyl-CoA reductases are tested for their ability to convert octanoic acid to 1-octanol. (B) DE cells carrying pACYC-^{Mt}FadD6 and different acyl-CoA reductases in a pBTRK plasmid were tested to determine an optimal ACR. Cells were grown in LB media with 500 mg/L octanoic acid (see materials and methods), and the 1-octanol production was tracked over time. ^{Ma}ACR, ^{Mt}ACR and ^{Mb}ACR were the best performers with similar peak production.

Assembly of the full pathway

With all enzymes selected, we assembled the complete pathway in order to test the ability of *E. coli K12 MG1655* to produce 1-octanol from unrelated carbon sources. We initially expressed each enzyme from separate plasmids in *E. coli* strain DE (Δ*fadD* Δ*fadE*). The thioesterase CpFatB1* was expressed from pTRC99a, the ACS ^{Mt}FadD6 from pACYC, and the ACR ^{Ma}ACR from pBTRK (see Tables S1 and S2 for a detailed description of strains and plasmids). A control strain lacking the ACS expression vector was created to track free fatty acid accumulation as a baseline. Cultures of *E. coli* DE containing each plasmid were grown in shake flasks containing 50 mL MOPS rich media and samples were collected at 12 h, 18 h, and 24 h to track accumulation of products as well as evaporation effects. Control strains lacking an ACS produced 1.1 g/L octanoic acid after 18 h under these conditions (**Figure 4A**) and did not produce any alcohols (**Figure 4B,** first panel). The cells also produced C_{16} and C_{18} saturated and unsaturated fatty acids at ratios consistent with prior *E. coli* studies (Lennen, 2010; Lennen, 2013). It should be noted that the acid-catalyzed methylation reaction used to produce quantifiable fatty acid methyl-esters also trans-esterifies lipid esters. When the ^{Mt}FadD6 ACS was co-expressed in the DE strain, cultures accumulated 1.0 g/L 1-octanol after 18 h and minor amounts of other chain-lengths (**Figure 4B,** first panel). Interestingly, we observed a similar strain (*ΔaraBAD ΔfadD*) carrying the same pTRC99a-CpFatB1* expression vector and expressed under the same conditions (**Figure S2C**) produce 50% more octanoic acid. This suggests that there may be a burden associated with carrying three plasmids in the cell.

For this reason, we created strains to integrate all genes to the chromosome of *E. coli*. We constructed *E. coli* NHL19 (*ΔfadE, ΔfadD::*P_{TRC}*CpFatB1*)* and repeated the experiment as described above with pTRC99a empty plasmid taking the place of the pTRC99a-CpFatB1* plasmid. NHL19 lacking ^{Mt}FadD6 ACS accumulated 0.9 g/L octanoic acid after 18 h (**Figure 4A,** second panel) while not producing any alcohols (**Figure 4B** second panel). When the ^{Mt}FadD6 ACS was included, NHL19 accumulated 0.6 g/L 1-octanol after 18 h and minor amounts of other chain-lengths (**Figure 4B,** second panel). Interestingly, the 1-octanol titers observed were substantially less than the octanoic acid titers produced in the corresponding control strains. We suspect that this difference is caused by a combination of factors including 1-octanol evaporation and the extra metabolic cost (1 ATP and 2 NADPH) required to convert octanoic acid to 1-octanol.

Next, we moved the ^{Mt}*FadD6* ACS to the chromosome, generating NHL22 (*ΔfadE, ΔfadD::*P_{TRC}CpFatB1*, *ΔackA-pta::*P_{TRC}-^{Mt}*FadD6*). We repeated the production experiment as described above with empty pACYC and with pACYC-^{Mt}FadD6 in order to compare chromosomal expression against the higher copy expression vector in the same strain. Interestingly, strain NHL22 maintained the same level of 1-octanol production made by NHL19 (**Figure 4B,** third panel). Additional plasmid expression of ^{Mt}FadD6 did not increase the performance of the strain in terms of 1-octanol production after 18 h, albeit the earlier 12 h time point shows less 1-octanol. Moreover, little accumulation of octanoic acid was observed in this strain, suggesting that chromosomal expression of ACS was sufficient to process the octanoic acid available. Up to this point, these strains maintained three plasmids (empty vectors when expression was tested from the chromosome). However, as more genes were moved to the chromosome (strains NHL23 and NHL24) the plasmid load was reduced.

Last, we moved *Ma*ACR to the chromosome creating a fully chromosomal 1-octanol production strain, NHL23 (*ΔfadE, ΔfadD::*P$_{TRC}$CpFatB1*, *ΔackA-pta::P*$_{TRC}$^{Mt}*FadD6, ΔFadBA::P*$_{TRC}$^{Ma}*ACR*). This strain was tested with an empty pBTRCK and with additional pBTRCK-^{Ma}ACR expression vector (**Figure 4A-B,** fourth panel). NHL23 produced over 1 g/L 1-octanol and 0.6 g/L of octanoic acid. Additional expression of ^{Ma}ACR in NHL23 did not increase octanol titers. Given this data, we hypothesized that ACS activity was a bottleneck in the conversion of octanoic acid to octanol. Therefore, we introduced a second copy of the ACS expression cassette on the chromosome, creating strain NHL24 (*ΔfadE, ΔfadD::*P$_{TRC}$CpFatB1*, *ΔackA-pta::P*$_{TRC}$-^{Mt}*FadD6, ΔpoxB::P*$_{TRC}$-^{Mt}*FadD6, ΔFadBA::P*$_{TRC}$^{Ma}*ACR*). This strain accumulated 1.3 g/L 1-octanol after 18 h, with lower accumulation of octanoic acid (**Figure 4A-B,** fifth panel). It is interesting to note that the 1-octanol titer produced by *E. coli* NHL24 was substantially higher than that of NHL23. The major difference between these strains is the removal of all plasmids, which likely removed a substantial metabolic burden from the cell as seen elsewhere (Glick, 1995; Wu et al., 2016). On the other hand, we attribute the reductions in titer observed in the 24h time points to be due to evaporation. Moreover, we speculate that combining these strains with a fermentation system to capture all the 1-octanol being produced will result in higher observed titers. However, efforts to develop such a system have proven difficult. In aerated bioreactors, gas flow strips volatile products from the aqueous fermentation broth at higher rates than evaporation from shake flasks. Capture of stripped products can theoretically be accomplished by condensation, adsorption on solid resins, or absorption by chilled solvents. We have made multiple attempts to capture the 1-octanol using small scale condensation or absorption systems but technical challenges such as the high aeration rate, humidity of the off-gas stream, clogged lines due to ice formation, absorber temperature, and the composition of the absorber organic layer have led to failures in each case (not shown). Nevertheless, shake-flask titers reported here represent a 2.3-fold increase to the highest 1-octanol titer reported in the literature (see **Table S3** for a summary of previous medium-chain alcohol efforts). Importantly, NHL24 is a fully chromosomally integrated strain, a feature that distinguishes it from previous approaches to produce 1-octanol.

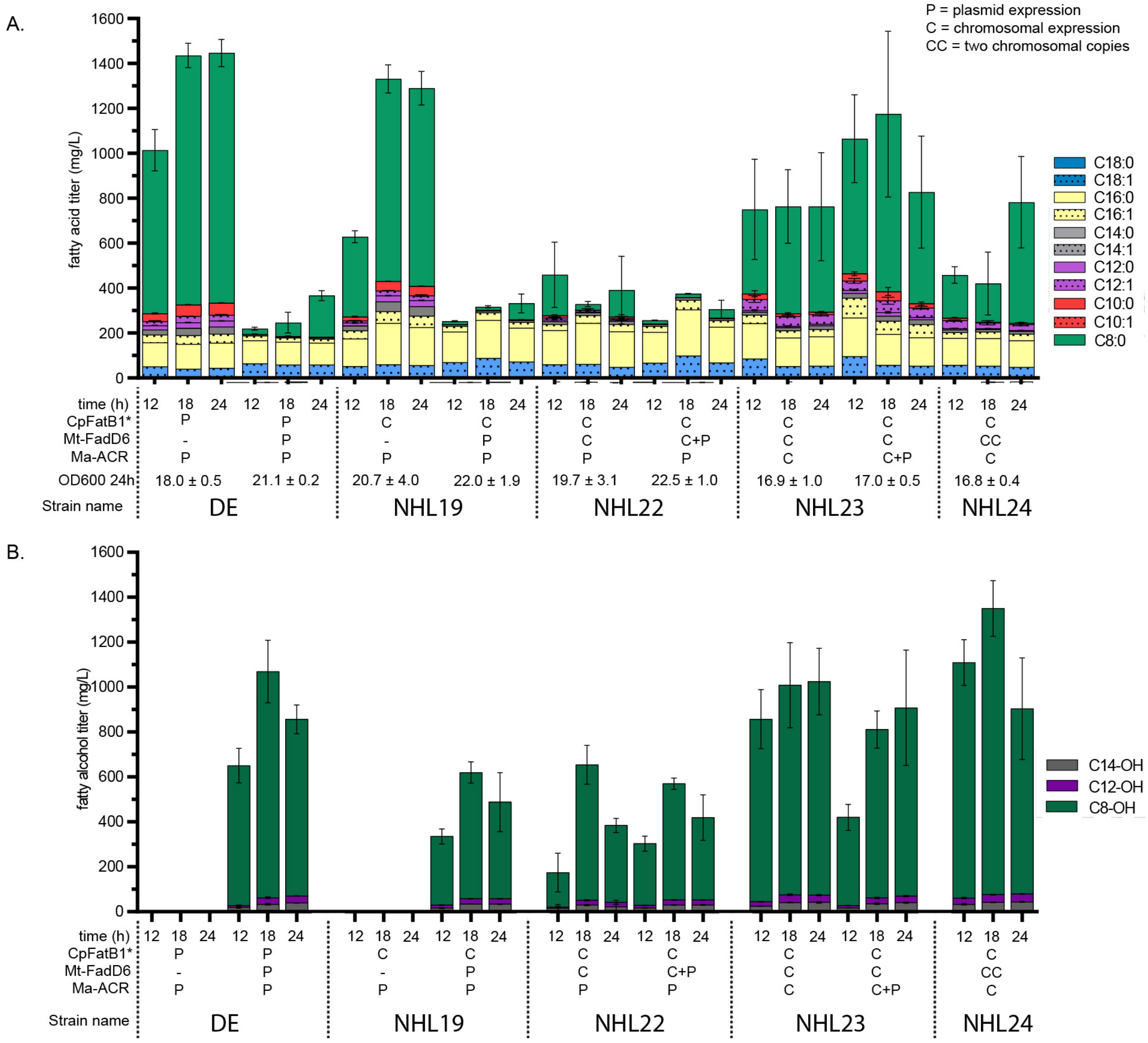

Figure 4. Developing plasmid-free 1-octanol producing strain

Strains containing 1-octanol pathway enzymes are first expressed exclusively from plasmids (strain DE, first panel) and then the genes were added systematically into the chromosome (strains NHL19 and NHL22; second and third panels, respectively) until reaching fully chromosomally expressed strain (NHL23, fourth panel). Finally, a second copy of *Mt-FadD6* was added to the chromosome (NHL24, fifth panel) (A) indicates the fatty acid, and (B) fatty alcohol titer for strains DE (*ΔfadD ΔfadE*) (first panel); NHL19 (*ΔfadE ΔfadD::*P_{TRC}CpFatB1*) (second panel); NHL22 (*ΔfadE ΔfadD::*P_{TRC}CpFatB1* *ΔackA-pta::*P_{TRC}-^{Mt}*FadD6*) (third panel); NHL23 (*ΔfadD::*P_{TRC}CpFatB1* *ΔfadE ΔackA-pta::*P_{TRC}-^{Mt}*FadD6 ΔFadBA::*P_{TRC}^{Ma}*ACR*) (fourth panel); and NHL24 (*ΔfadD::*P_{TRC}CpFatB1* *ΔfadE ΔackA-pta::*P_{TRC}-^{Mt}*FadD6 ΔpoxB::*P_{TRC}-^{Mt}*FadD6 ΔFadBA::*P_{TRC}^{Ma}*ACR*) (fifth panel). Chromosomally expressed genes are indicated by a 'C' and plasmid expressed are denoted by a 'P'. Genes being expressed both chromosomally and in plasmid are denoted as 'C+P'. OD_{600} for 24 h time points are shown as well.

Conclusions

In this work, we demonstrated 1-octanol production from simple sugars using a set of *E. coli* strains engineered to direct flux through fatty acid biosynthesis, a C_8-specific thioesterase, an acyl-CoA synthetase, and an acyl-CoA reductase/aldehyde reductase. Our experiments determined that 1.) MG1655 was a superior base strain to LS5218 for aerobic 1-octanol production; 2.) deletion of acetate and lactate pathways had little impact on octanoic acid titers, likely because they are not the current bottlenecks of the pathway; 3.) expression of the thioesterases (CpFatB1*) needed to be low, 4.) two heterologous acyl-CoA synthetases from *P. putida* and *M. tuberculosis* were superior to native *E. coli* FadD for activating octanoic acid, and 5.) the pathway can be expressed fully from the chromosome. Our best plasmid-free strain produced 1.3 g/L 1-octanol in shake flasks. In addition to the strain development findings, we alleviated challenges associated with quantifying octanol by adding an organic overlay and internal standards. Future work will focus on the scaling up of this system to bioreactor experiments as well as developing a 1-octanol capture mechanism.

Author Contributions

NJHL and BFP designed the study. NJHL, TRS, KX, MAJ built strains, performed experiments and collected data. NJHL and BFP analyzed the data and wrote the original manuscript. NJHL, MAJ, and BFP edited the manuscript.

Declaration of competing interest

The authors declare no competing interests.

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Supplementary Information for

Production of 1-octanol in *Escherichia coli* by a high flux thioesterase route

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Table S1: Strains used this study

Strain	Genotype	Source
E. coli K12 MG1655	F^- λ^- $ilvG^-$ rfb-50 rph-1	CGSG
E. coli BL21 DE3	F^- *hddS gal lacI::PlacUV5::T7 gene1*	Studier et al., 1986
BW25141	*lacIq rrnB3 ΔlacZ4787 hsdR514* DE*(araBAD)567* DE*(rhaBAD)568 ΔphoBR580 rph-1 galU95 ΔendA9 uidA(ΔMluI)::pir(wt) recA1*	Haldimann et al., 2001
RL08ara	K-12 MG1655 *ΔaraBAD ΔfadD*	Lennen al., 2010
araBAD	K-12 MG1655 *ΔaraBAD*	Agnew et al., 2012
NHL17	K-12 MG1655 *ΔaraBAD ΔfadD::*P_{TRC}CpFatB1*-*lacI*	Hernandez-Lozada et al., 2018
NHL09	K-12 MG1655 *ΔaraBAD ΔfadD ΔackA-pta*	This work
NHL10	K-12 MG1655 *ΔaraBAD ΔfadD ΔackA-pta ΔpoxB*	This work
NHL11	K-12 MG1655 *ΔaraBAD ΔfadD ΔackA-pta ΔpoxB ΔldhA*	This work
MHS04	K-12 MG1655 *ΔfadR ΔfadD*	Youngquist et al., 2013
DE	K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD*	Agnew et al., 2012
NHL19	K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD::*P_{TRC}CpFatB1*-*lacI*	This work
NHL22	K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD::*P_{TRC}CpFatB1*-*lacI*, *ΔackA-pta::*P_{TRC}-^{Mt}*FadD6-lacI*	This work
NHL23	K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD::*P_{TRC}CpFatB1*-*lacI*, *ΔackA-pta::*P_{TRC}-^{Mt}*FadD6-lacI, ΔFadBA::*P_{TRC}^{Ma}*ACR-lacI*	This work
NHL24	K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD::*P_{TRC}CpFatB1*-*lacI*, *ΔackA-pta::*P_{TRC}-^{Mt}*FadD6--lacI, ΔpoxB::*P_{TRC}-^{Mt}*FadD6, ΔFadBA::*P_{TRC}^{Ma}*ACR-lacI*	This work
LS5218	*E. coli* LS5218	Rand et al., 2017
NHL25	LS5218 *ΔfadD*	This work
NHL26	LS5218 *ΔfadD::*P_{TRC}CpFatB1*-*lacI*	This work

Table S2: Plasmids used in this study

Plasmid	Genotype	Source
pBTRKtrc	P_{trc} promoter, pBBR1 origin, Kan^R	Youngquist et al., 2013
pTRC99a	P_{trc} promoter, pBR322 origin, Amp^R	Amann et al., 1988
pACYC	P_{trc} promoter, pACYC origin, Cm^R	Youngquist et al., 2013
pKD13	R6Kγ origin, Amp^R	Haldimann, et al., 2001
pMP11	pKD46 with constitutively expressed *cas9*, P_{ara}λ-Red, P_{TO} guide RNA targeting the pBR322 origin of replication, Amp^R	Mehrer et al., 2018
pgRNA	Constitutively expressed guide RNA targeting chromosomal locus of gene integration or knockout, pBR322 origin, Cm^R or Kan^R	Mehrer et al., 2018
pBTRK-CpFatB1*	pBTRKtrc with mutant CpFatB1.2-M4-287 sequence	This work
pTRC99a-CpFatB1*	pTRC99a with CpFatB1.2-M4-287 sequence	This work
pBTRK-Ma-ACR	pBTRKtrc with *Marinobacter aquaeolei* VT8 acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Mb-ACR	pBTRKtrc with *Marinobacter BSs20148* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Mt-ACR	pBTRKtrc with *Methylibium Sp. T29* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Na-ACR	pBTRKtrc with *Nocardia asteroids* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Ca-adhE2	pBTRKtrc with *Clostridium acetobutylicum* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Ra-ACR	pBTRKtrc with *Rhodococcus aetherivorans* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Mc-ACR	pBTRKtrc with *Mycobacterium chlorophenolicum* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Mh-ACR	pBTRKtrc with *Mycobacterium hassiacum* acyl-CoA reductase	Mehrer et al., 2018
pBTRK-Rc-ACR	pBTRKtrc with *Rhodococcus sp. PBTS 2* acyl-CoA reductase	Mehrer et al., 2018
pACYC-Mt-FadD6	pACYC with *Mycobacterium tuberculosis fadD6* acyl-CoA synthetase	Youngquist et al., 2013

pACYC-Pp-0763	pACYC with *Pseudomonas putida Pp-0763* acyl-CoA synthetase	Youngquist et al., 2013
pTRC99a-fadD	pTRC99a with *E. Coli fadD* acyl-CoA synthetase	Youngquist et al., 2013
pKD13-fadD-CupTE2M4287_15KAU	pKD13 with P_{TRC}*CpFatB1** and *lacI*, 500 base pairs of homology to *E. coli* K12 MG1655 *fadD* chromosomal locus	This work
pKD13-fadBA-loci-MaACR	pKD13 with P_{TRC}*MaACR* and *lacI*, 500 base pairs of homology to *E. coli* K12 MG1655 *fadBA* chromosomal locus	This work
pKD13-ackApta-loci-FadD6_MaxRBS	pKD13 with P_{TRC}*fadD6* with maximum RBS strength as calculated by the RBS calculator (Salis et al. 2009) and *lacI*, 500 base pairs of homology to *E. coli* K12 MG1655 *ackApta* chromosomal locus	This work
pKD13-poxB-loci-FadD6_MaxRBS	pKD13 with P_{TRC}*fadD6* with maximum RBS strength as calculated by the RBS calculator (Salis et al. 2009), 500 base pairs of homology to *E. coli* K12 MG1655 *poxB* chromosomal locus	This work

Table S3: Medium-chain fatty alcohols literature review

Compound produced	strain	Media/fermentator	Titer (mg/L)	Temp (C)	Pathway elongation	Termination enzymes			
						Thioesterase	ACS	CAR	ACR
C8OH	Ec MG1655 fadR atoC(con) ΔarcA ΔcrpːːcrpΔ* ΔadhE Δpta ΔfrdA ΔfucO ΔyqhD ΔfadD fadBA betA	M9 2% glucose with vitamins / shake flasks	~80	37	rβOX	N/A	N/A	N/A	N/A
C8OH	Ec BW25113 F0 [traD36, proABþ, lacIq ZΔM15 (tetR)] ΔldhA ΔadhE ΔfrdBC Δpta	Terrific broth, 2% glucose / 10mL tubes	~65	30	"+1"	N/A	N/A	N/A	Ca funct
C12-C16 OH	Ec BL21 (DE3)	M9 2% glucose / falcon tubes	~ 363 (total) ~50% C12OH	30	FAB	Ec TesA'	N/A	Mm	N/A
C12-C14 OH	MG1655 ΔaraBAD ΔfadE::trcBTE ΔfadAB::trcBTE ΔackApta::trcBTE Φ(PTrc-fadD)	MOPS minimal media, phosphate limited 0.7% glucose / 1L bioreactor	1650 (total); ~1200 C12OH	30	FAB	UcFatB1	Ec	N/A	Ma D
C8OH	Ec BL21 (DE3)	M9 glucose with vitamins / Shake flasks	100	30	FAB	Ec TesA'	N/A	Mm	N/A
C11-C15 odd chain OH	Ec BL21 (DE3)	Mineral medium 1.5% glycerol 5 g/L yeast extract with more glycerol + yeast extract added in fed batch; 2.5L	1950 (total); 1290 C13OH, 362 C11OH	30	FAB	Ec TesA'	N/A	N/A	N/A
C10OH	Yl W29 Δpex10	Low-nitrogen medium 5% glucose, 1.7 g/L yeast nitrogen base without amino acids, 0.15 g/L yeast extract / shake flasks	550	30	FAB	CpFatB1	Yl	N/A	At Du
C6-C14 OH	Ec LS5218 ΔfadE ΔatoC ΔldhA ΔackApta ΔadhE ΔpoxB ΔfrdABCD ΔydiO ΔfadBA ΔfadIJ ΔfadD	LB + 1% glucose / Hungate tubes 10 mL	1800 (total), ~200 C8OH	30	rβOX	N/A	N/A	N/A	Ma D
C8-C10 OH	Synechocystis sp. PCC 6803	BG11 medium without cobalt; shake flasks	~54 C8OH, ~40 C10OH	30	FAB	At tes3	N/A	Mm	N/A
C8OH	Ec BW25113	M9 minimal media, 25 mL shake flasks 48h	558	30	FAB	CpFatB1*	N/A	Mm	N/A
	Synechocystis sp. PCC 6803	BG11 medium without cobalt; shake flasks	~340			CaFatB1*			
C8OH	Ec K-12 MG1655 *ΔaraBAD, ΔfadE, ΔfadD::*P_{TRC}CpFatB1*-*lacI*, *ΔackA-pta::*P_{TRC}*-Mt-FadD6--lacI, ΔpoxB::*P_{TRC}*-Mt-FadD6, ΔFadBA::*P_{TRC}*Ma-ACR-lacI*	MOPS rich media + 2% glycerol / shake flasks	1273	30	FAB	CpFatB1*	Mt	N/A	Ma D

Abbreviations
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Ec: Escherichia coli; Ma: Marinobacter aquaeolei; Mt: Mycobacterium tuberculosis; Mm: Mycobacterium marinum; Bs: Bacillus subtilis; 6803: Synechocystis species PCC 6803, slr1192; At: Arabidopsis oleovorans; Ca: Clostridium acetobutylicum; At; anaerococcus tetradius

Bs Sfp is required for activation of CAR enzyme
rβOX: reverse B-oxidation; FAB: fatty acid biosynthesis

Supplementary Figures

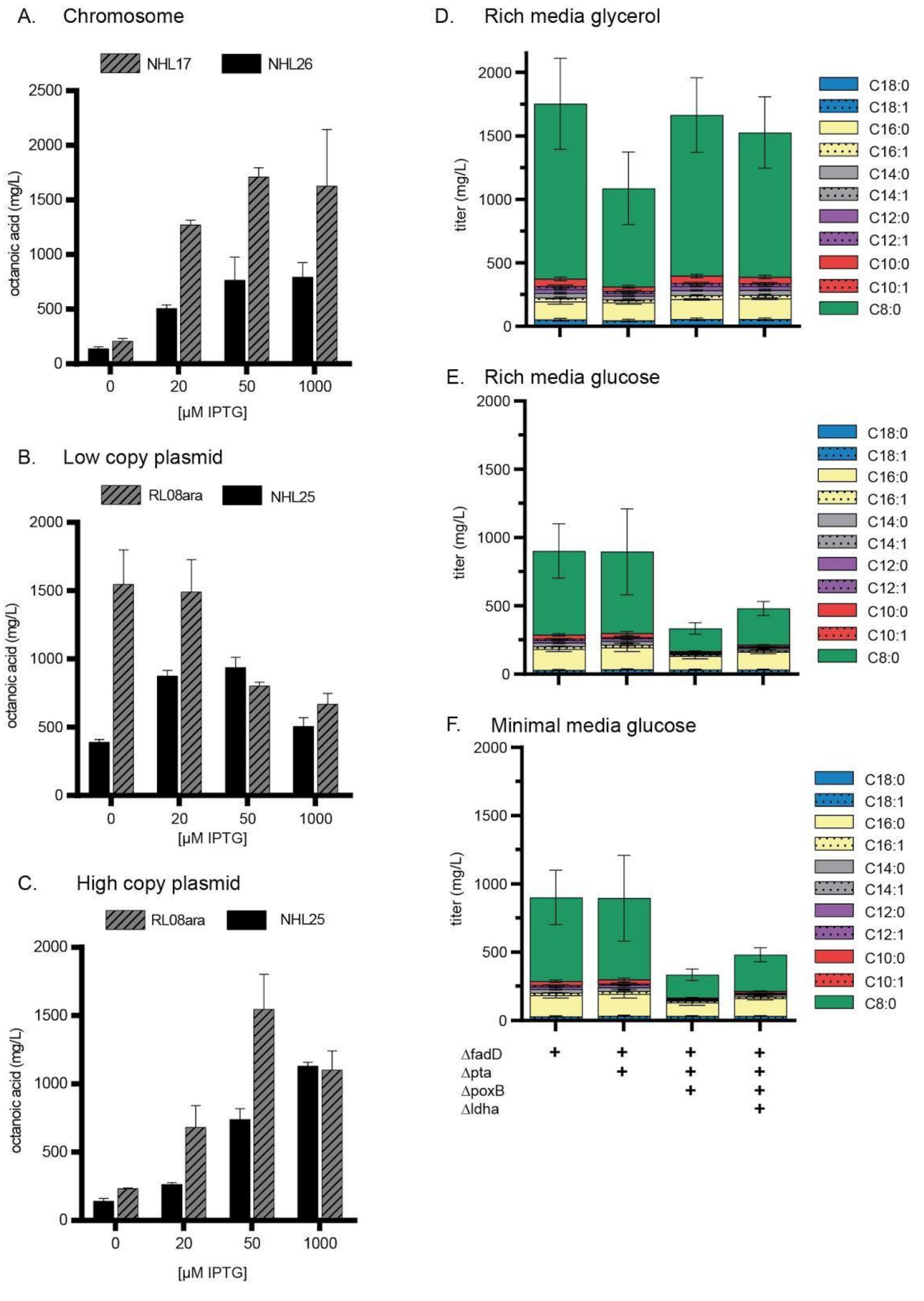

Figure S1. Octanoic acid production of various fermentation knockout strains.

(A) Fatty acid production at different induction levels for strains NHL17 (MG1655 *ΔfadD::PtrcCpFatB1**) and NHL26 (LS5218 *ΔfadD::PtrcCpFatB1*)* which contain chromosomal integrations of CpFatB1*. (B-C) Fatty acid production at different induction levels for strains RL08ara (MG1655 *ΔfadD*) and NHL25 (LS5218 *ΔfadD*) transformed with either a low copy plasmid pBTRK-CpFatB1* with a strong RBS (B) or high copy plasmid pTRC99a- CpFatB1* with a weak RBS (C) (see discussion for details). (D-F) MG1655 ΔfadD with additional fermentation pathway deletions carrying pTRC99a- CpFatB1* and tested in total three media conditions, rich media with glycerol (D), rich media with glucose (E), and MOPS minimal media phosphate limited with glucose (F).

Addressing Octanol Quantification Challenges

In the “1-octanol evaporation” section of the main text we discussed how 1-octanol quantification in the presence of dodecane had an underestimation bias. This was a phenomenon that was observed only when the samples were taken out of a shake flask into a glass centrifuge tube for further processing. It should be noted that a media:dodecane mixture has the characteristic of separating into two layers within several seconds, with the top layer being dodecane rich and the bottom media rich. This makes sampling of the culture challenging as the shake flask has to be well mixed for homogeneous sample extraction. We hypothesized that our sampling process might be behind the underestimations if the sample taken was slightly biased towards taking more of the media-rich layer than the organic layer. Although we routinely add internal standards to account for these types of losses during the sample processing our protocols were designed to add the internal standard to the sample once it is in the centrifuge tube. This internal standard practice would not be able to correct for any losses prior to adding the sample to the tube (something that is typically not an issue in one-phase systems) and thus we hypothesized that adding the internal standard to the shake flask would correct this issue. To test this, we created an 10X concentrated internal standard such that upon adding the appropriate volume to the shake flask would result in the same concentration of internal standard in the media as in the internal standard added to the tube. We tested side-by-side samples of media containing known amounts of 1-octanol ranging from 50 to 2000 mg/L with internal standard added directly to the shake flask versus internal standard added to the tube post sampling (**Figure S3**). When the internal standard is added directly to the shake flask the quantified 1-octanol line is closer to the 1:1 line at any given concentration tested (**Figure S3A**). Percent error difference from the expected values was calculated for each sample and shows a large error rate (up to 40%) for the samples when the internal standard was added to the sample in the tubes versus errors ranging from <1% to 12% for the samples with internal standard added to the shake flask (**Figure S3B**). Based on these results we decided to modify the sampling protocol to add the internal standard to the shake flask prior to taking samples for all the samples that involved dodecane as an organic layer.

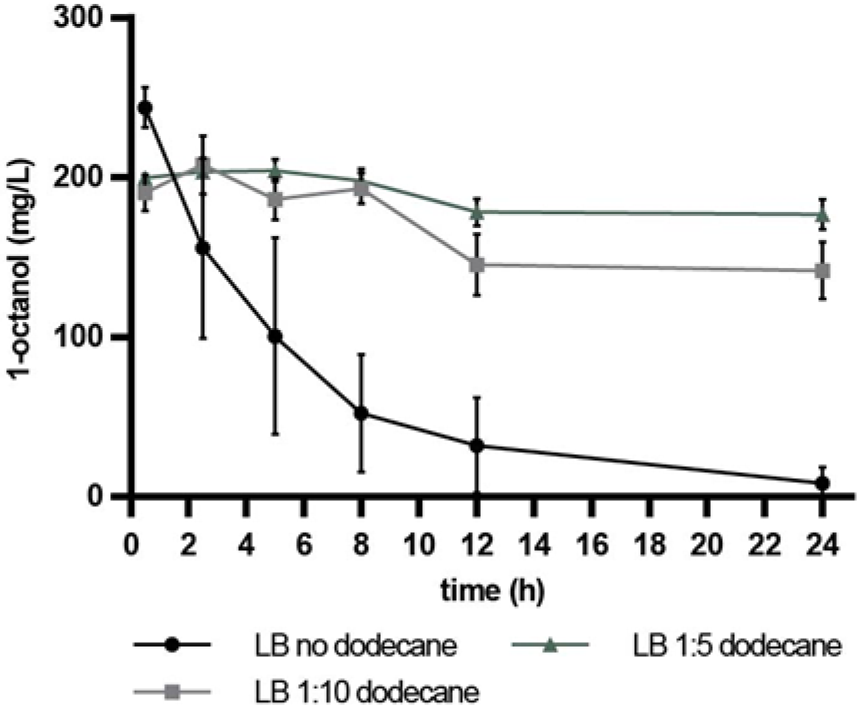

Figure S2. **Evaporation of 1-octanol is significant and can be reduced by the presence of dodecane.**

1-Octanol evaporation in 250 mL shake flasks was tracked over time to study evaporation behavior in aerobic conditions. When no dodecane was added, 250 mg/L of 1-octanol is lost to evaporation over a period of 24 hr. Addition of 1:5 and 1:10 volumetric ratios of dodecane reduced evaporation rates, however, the observed titers of 1-octanol in the presence of dodecane were underestimated (~ 75% of expected value).

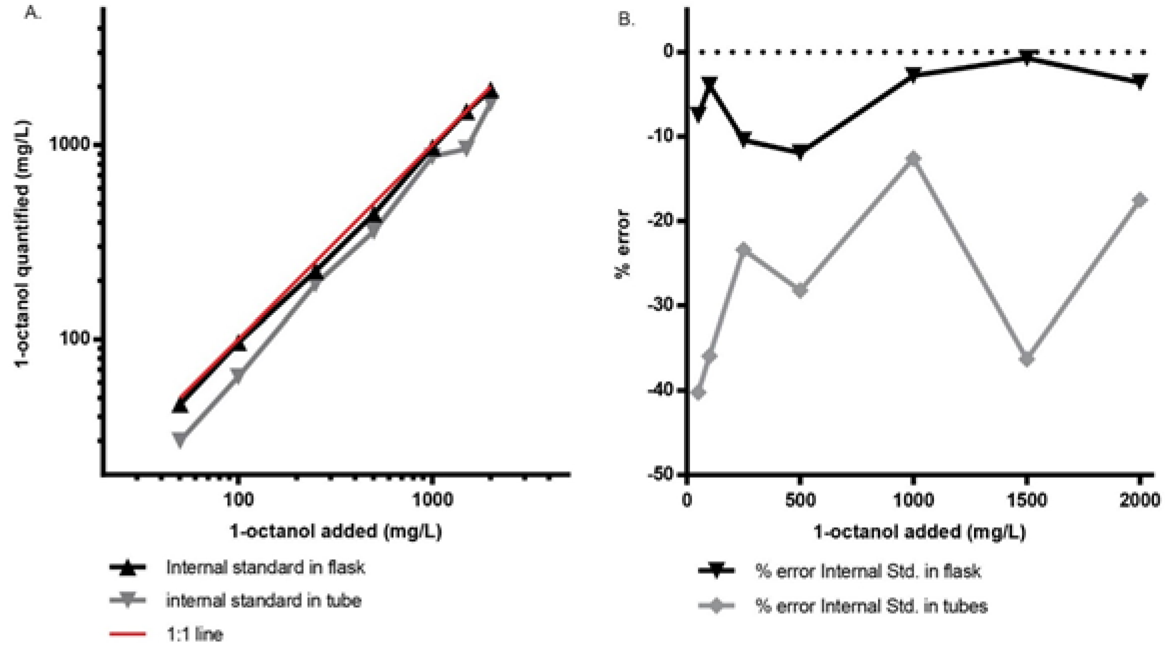

Figure S3. Quantification of known amounts of 1-octanol from shake flasks containing 1:5 dodecane:media mixture in scenarios where an internal standard is added to the shake flask directly before sampling or to the sample tube after sample is taken. (A) shows the 1-octanol quantified vs the 1-octanol added to the media. (B) shows the error associated with each measurement. In all cases, we found that adding the internal standard to the shake flask before sampling was more accurate for quantification of the octanol in the media.

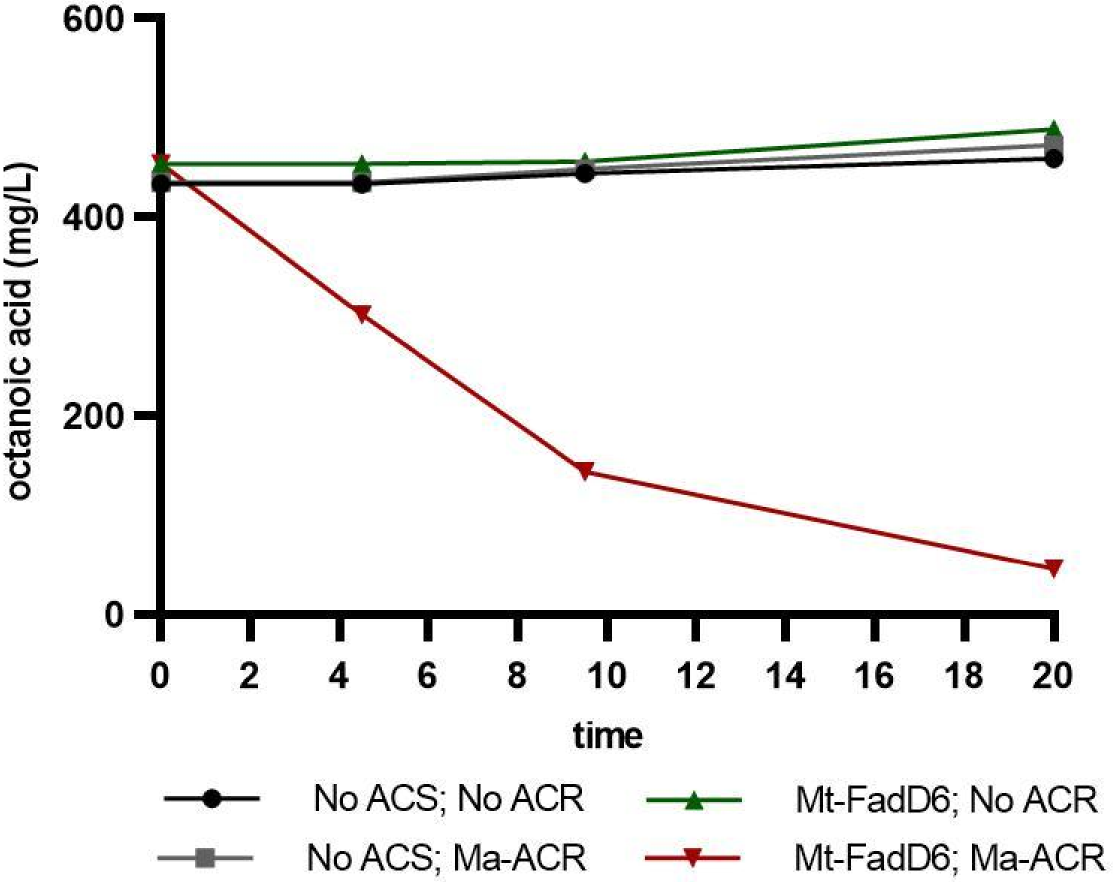

Figure S4. Before testing 1-octanol production from sugars we decided to test production from fed octanoic acid in order to ensure that the acyl-CoA reductase was sufficiently active octanoyl-CoA. To do this we started from a MG1655 *ΔfadD ΔfadE* strain which prevents β-oxidation from metabolizing any acyl-CoA into enoyl-CoA and has only ACS activity if the ACS is provided in a plasmid. This strain was transformed with two plasmids containing the ACS (Mt-FadD6) and the ACR from *Marinobacter aquaeolei* (Ma-ACR) (Willis et al., 2011). Three controls were used: (1) plasmids containing the ACS but not the ACR (empty plasmid was used when an enzyme was not expressed), (2) plasmids containing the ACR but not the ACS, and (3) plasmids containing neither the ACS nor ACR. Each strain/plasmid combination was grown in shake flasks containing 50 mL LB media + 500 mg/L octanoic acid (added immediately after induction) and samples were taken over time to track the consumption of octanoic acid. The octanoic acid was consumed only in cells containing both the acyl-CoA synthetase and the acyl-CoA reductase. Moreover, Ma-ACR is able to turnover most of the octanoyl-CoA by 20 h. All other cases resulted in unconsumed substrate. It should be noted that the acid catalyzed derivatization method used for the quantification of free fatty acids cannot differentiate between the free acids and the CoA species as it methylates both. Thus, the sample containing ACS but no ACR, which is known from **Figure 2** to have activity on octanoic acid, appears to not have consumed the free fatty acid.

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