Substantial improvements in methyl ketone production in *E. coli* and insights on the pathway from *in vitro* studies

Ee-Been Goh^{† 1,3}, Edward E. K. Baidoo^{1,3}, Helcio Burd^{1,3},
Taek Soon Lee^{1,3}, Jay D. Keasling^{1,3,4}, Harry R. Beller*^{1,2}

¹Joint BioEnergy Institute, 5885 Hollis Avenue, Emeryville, CA 94608

²Earth Sciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

³Physical Biosciences Division, Lawrence Berkeley National Laboratory, Berkeley, CA 94720

⁴Departments of Chemical & Biomolecular Engineering and of Bioengineering, University of
California, Berkeley, CA 94720

***Corresponding author:**

Harry R. Beller
Joint BioEnergy Institute
5885 Hollis Avenue
Emeryville, CA 94608

E-mail HRBeller@lbl.gov
Phone (510) 486-7321
Fax (510) 486-5686

Abstract

We previously reported development of a metabolic pathway in *E. coli* for overproduction of medium-chain methyl ketones (MK), which are relevant to the biofuel and flavor-and-fragrance industries. This MK pathway was a re-engineered version of β -oxidation designed to overproduce β -ketoacyl-CoAs and involved overexpression of the *fadM* thioesterase gene. Here, we document metabolic engineering modifications that have led to a MK titer of 3.4 g/L after ~45 hr of fed-batch glucose fermentation and attainment of 40% of the maximum theoretical yield (the best values reported to date for MK). Modifications included balancing overexpression of *fadR* and *fadD* to increase fatty acid flux into the pathway, consolidation of the pathway from two plasmids into one, codon optimization, and knocking out key acetate production pathways. *In vitro* studies confirmed that a decarboxylase is not required to convert β -keto acids into MK and that FadM is promiscuous and can hydrolyze several CoA-thioester pathway intermediates.

Keywords

Methyl ketones; fatty acid; biofuels; FadM; beta-oxidation; acetate

1. Introduction

Aliphatic methyl ketones are naturally occurring compounds (Forney and Markovetz, 1971) that can serve several different commercial applications. For example, medium-chain methyl ketones such as 2-undecanone and 2-tridecanone are used in the flavor and fragrance industry (Longo and Sanromán, 2006) and were recently reported as promising diesel fuel blending agents (Goh et al., 2012). Goh and co-workers demonstrated the production of over 380 mg/L of C₁₁ to C₁₅ methyl ketones from glucose (0.2%, in tryptic soy broth) in an engineered *E. coli* DH1 strain, which represented a 700-fold increase in titer relative to a fatty acid-overproducing strain (Goh et al., 2012). The major features of this *E. coli* strain were a re-engineered β -oxidation pathway designed to overproduce β -ketoacyl-CoAs and overexpression of the *fadM* gene encoding a native thioesterase. Subsequently, Park and co-workers (Park et al., 2012) described engineering of an *E. coli* MG1655 strain that was able to produce 14 mg/L of methyl ketones (2% glucose) when overexpressing a β -ketoacyl-ACP thioesterase (*shmks2*) and a β -keto acid decarboxylase (*shmks1*) from *Solanum habrochaites* (wild tomato) and when *adhE*, *ldhA*, *poxB*, and *pta* were deleted from the chromosome. Batch incubations of the best strain under optimized growth conditions with 5% glucose resulted in a methyl ketone titer of ca. 500 mg/L. In 2013, Müller et al. (2013) transferred the methyl ketone pathway of Goh and co-workers into *Ralstonia eutropha*, a bacterium that can grow chemolithoautotrophically with CO₂ and H₂ as the sole carbon source and electron donor, respectively. *R. eutropha* was reported to produce up to 180 mg/L of methyl ketones under such chemolithoautotrophic growth conditions (Müller et al., 2013). Lan et al. (2013) produced 2-pentanone (for possible biomedical application) in *E. coli* strain BW25113 by using a metabolic pathway that, unlike the other studies cited above, did not rely on fatty acid precursors. More specifically, the pathway

involved a biosynthetic thiolase reaction catalyzed by BktB to condense acetyl-CoA with butyryl-CoA, forming β -ketohehexanoyl-CoA, which was converted by a PcaIJ-catalyzed CoA-transferase reaction to β -ketohehexanoic acid, which was then converted to 2-pentanone by acetoacetate decarboxylase (Adc). The best reported 2-pentanone titer in that study was 240 mg/L using 4% glucose in Terrific Broth (Lan et al., 2013). Another pathway for the synthesis of short-chain methyl ketones was recently reported by Yoneda et al. (2014) for the production of 2-butanone in *E. coli*. The pathway builds on the 2,3-butanediol biosynthetic pathway and uses a B₁₂-dependent glycerol dehydratase from *Klebsiella pneumoniae* to convert 2,3-butanediol to 2-butanone. Further co-overexpression of the *orfz* and *orf2B* genes, which encode the co-enzyme B₁₂ reactivator, resulted in a 2-butanone titer of 151 mg/L in minimal medium containing 3.6% glucose (Yoneda et al., 2014).

In this paper, we provide insights from further characterization of the methyl ketone pathway first reported by Goh et al. (2012) using *in vitro* studies with purified pathway enzymes. In addition, we detail the engineering of a series of pathway and host modifications that improved the titer of the best methyl ketone-producing *E. coli* strain reported by Goh et al. (2012) more than 160-fold in minimal medium to 1.4 g/L (1% glucose, shake flasks), which represents 40% of the maximum theoretical yield. In addition, using fed-batch fermentation, we were able to achieve a methyl ketone titer of 3.4 g/L after 45 hr of production.

2. Materials and Methods

2.1. Bacterial strains, plasmids, oligonucleotides, and reagents

Bacterial strains and plasmids used in this study are listed in Table 1. Strains and plasmids along with their associated information (annotated GenBank-format sequence files) have been deposited in the public version of the JBEI Registry (<https://public-registry.jbei.org>;

entries JPubl_XXXX [to be added in proofs]) and are physically available from the authors and/or addgene (<http://www.addgene.org>) upon request. Restriction enzymes were purchased from Thermo Scientific (Waltham, MA), and Phusion DNA polymerase and T4 ligase were from New England Biolabs (Ipswich, MA). Plasmid extractions were carried out using the QIAGEN (Valencia, CA) miniprep kits. Oligonucleotide primers were designed using the web-based PrimerBlast program (http://www.ncbi.nlm.nih.gov/tools/primer-blast/index.cgi?LINK_LOC=BlastHomeAd) and synthesized by Integrated DNA Technologies (IDT), Inc (San Diego, CA) or Eurofins MWG Operon (Huntsville, AL). Primer sequences for amplification of *E. coli* DH1 and *Micrococcus luteus* ORFs are listed in Table S1. The coding sequences (CDS) of acyl-CoA oxidase (Mlut_11700, ACO) from *M. luteus* and FadD from *E. coli* were synthesized and codon optimized for expression in *E. coli* by GenScript, Inc. (Piscataway, NJ). Codon-optimized sequences are listed in Table S2.

2.2. Media and bacterial growth

E. coli was propagated aseptically as previously described (Sambrook et al., 1989). *E. coli* DH10B cells were used for cloning and LB medium was used for routine growth and propagation of strains. When required, antibiotics were added to the growth medium at the following final concentrations: chloramphenicol, 25 µg/mL; kanamycin, 50 µg/mL. TSB (containing 0.2% glucose) or M9-MOPS minimal medium [M9 medium supplemented with 75 mM MOPS, 2 mM MgSO₄, 1 mg/L thiamine, 10 nM FeSO₄, 0.1 mM CaCl₂, 56 mM NH₄Cl₂ and micronutrients including 3 mM (NH₄)₆Mo₇O₂₄, 0.4 mM boric acid, 30 mM CoCl₂, 15 mM CuSO₄, 80 mM MnCl₂, and 10 mM ZnSO₄] (Zhang et al., 2012a) with 10 g/L or 20 g/L glucose as the sole carbon source was used for production. For production in M9-MOPS minimal medium, strains were first adapted in the medium. Briefly, cells were grown in LB overnight and

diluted into M9-MOPS minimal medium at a starting OD₆₀₀ (optical density at 600 nm) of ~ 0.1. Cultures were grown until an OD₆₀₀ value of ~ 1.0 was reached (~ 24 hours) and re-diluted into fresh M9-MOPS medium as before. This was repeated two more times before the adapted cultures were stored as frozen glycerol stocks at -80°C until needed. When monitoring oxygen (O₂) consumption and carbon dioxide (CO₂) production during methyl ketone production, the Respiration Activity MONitoring System (RAMOS) system (Kuhner AG, Basel, Switzerland) was used. The determination of the oxygen transfer rate (OTR) and carbon dioxide transfer rate (CTR) were carried out as described by Anderlei et al. (2004).

2.3. Plasmid and strain construction for heterologous expression in *E. coli*

Cloning of *M. luteus* and *E. coli* genes into expression plasmids was carried out by PCR amplification with appropriate primers (Table S1) as previously described (Goh et al., 2012). Proper clone construction was confirmed by DNA sequencing, which was performed by Quintara Biosciences (Albany, CA). When required, expression of heterologous genes in constructs was confirmed by extraction of proteins, tryptic digestion, and analysis of the resulting peptides by electrospray ionization liquid chromatography-tandem mass spectrometry (LC/MS/MS)(QSTAR Elite Hybrid Quadrupole TOF, Applied Biosystems). In-frame chromosomal deletion of *E. coli* genes was carried out using the method of Datsenko and co-workers (Baba et al., 2006; Datsenko and Wanner, 2000).

2.4. *In vitro* assays with methyl ketone pathway enzymes

His₆-tagged acyl-CoA oxidase (ACO), His₆-tagged FadB, and His₆-tagged FadM were purified as previously described (Beller et al., 2010). All *in vitro* assays were conducted with 0.1 M potassium phosphate buffer (pH 7.5) containing 200 mg/L BSA and 1 mM DTT in 4-mL screw-cap glass vials (with polytetrafluoroethylene liners). To generate enoyl-CoAs, a reaction

containing 10 mM myristoyl-CoA (Sigma), 500 μ g (0.21 mM) of His₆-tagged acyl-CoA oxidase, and 0.1 mM FAD to a final volume of 350 μ L, was incubated on a stir plate at 30°C for 1.5 hr. Five microliters of catalase (Sigma) were then added to the reaction and incubated as before for another 30 min at 30°C to remove H₂O₂ generated by the acyl-CoA oxidase reaction. To proceed toward the latter steps of the pathway, 50 μ L of the acyl-CoA oxidase reaction mixture was added to a new 4-mL screw-cap glass vial containing (1) 25 μ g (0.28 mM) of His₆-tagged FadM, (2) 500 μ g (0.12 mM) of His₆-tagged FadB and 25 μ g (0.28 mM) of His₆-tagged FadM, or (3) 500 μ g (0.12 mM) of His₆-tagged FadB, 25 μ g (0.28 mM) of His₆-tagged FadM, and 6 mM NAD⁺, in a final volume of 250 μ L. In addition, a fourth reaction containing 2 mM of myristoyl-CoA and 25 μ g (0.28 mM) of His₆-tagged FadM was carried out to investigate hydrolysis of myristoyl-CoA by FadM. All four reactions were performed in duplicate. To stop the *in vitro* reactions at specific time points, 100 μ L of reaction mixture was removed and added to a glass vial containing an equal volume of methanol (LC-MS grade, Burdick and Jackson) and stored at -20°C until LC-TOF analysis. Methyl ketones in the reactions were analyzed by LC-APCI-TOF (liquid chromatography-atmospheric pressure chemical ionization-time of flight) mass spectrometry, while all other metabolites were analyzed by LC-ESI (electrospray ionization)-TOF mass spectrometry.

2.5. Analysis by LC-APCI-TOF and LC-ESI-TOF mass spectrometry

LC-APCI-TOF analyses were conducted at 55°C with a Kinetex XB-C18 column (100-mm length, 3-mm internal diameter, 2.6- μ m particle size; Phenomenex, Inc., Torrance, CA) using a 1200 Series HPLC (high-performance liquid chromatography) system (Agilent Technologies, CA). The mobile phase was composed of water (solvent A) and methanol (solvent B) (HPLC grade, Honeywell Burdick & Jackson, CA). Two-microliter samples were injected

and separated with the following gradient: 60% to 98% B for 3.47 min, held at 98% B for 5.2 min, decreased to 60% B for 2.42 min, and held at 60% B for a further 2.78 min. The total analysis run time was 13.87 min. A flow rate of 0.42 mL/min was used throughout. APCI-TOF MS conditions were described previously (Goh et al. 2012).

LC-ESI-TOF analyses were conducted at 55°C with the aforementioned Kinetex column using a 1200 Series HPLC system (Agilent Technologies, CA). The mobile phase was composed of 10 mM ammonium acetate (pH 6.75) in water (solvent A) and 10 mM ammonium acetate in methanol (solvent B) (HPLC grade, Honeywell Burdick & Jackson, CA). Two-microliter samples were injected and separated with the following gradient: 60% to 98% B for 3.47 min, held at 98% B for 5.2 min, decreased to 60% B for 0.33 min, and held at 60% B for a further 2 min. Metabolite separation was conducted *via* a flow rate of 0.42 mL/min from 0 to 8.67 min, which was increased to 0.65 mL/min from 8.67 to 9 min and held at this flow rate for an additional 2 min. The total analysis run time was 11 min. The HPLC system was coupled to an Agilent Technologies 6210 time-of-flight mass spectrometer (TOF MS) with a 1:5 post-column split. Nitrogen gas was used as both the nebulizing and drying gas to facilitate the production of gas-phase ions. The drying and nebulizing gases were set to 10 L/min and 30 psi, respectively, and a drying gas temperature of 325°C was used throughout. ESI was conducted in the negative-ion mode with a capillary voltage of 3.5 kV. MS experiments were carried out in the full-scan mode (m/z 170–1000) at 0.86 spectra per s for the detection of $[M - H]^-$ ions. The instrument was tuned for a range of m/z 50 – 1700. Prior to LC-ESI-TOF MS analysis, the TOF MS was calibrated with the Agilent ESI-L TOF tuning mix.

Data acquisition and processing were carried out by the Agilent MassHunter Workstation software. 2-Tridecanone, myristoyl-CoA, myristic acid, β -hydroxydecanoic acid (for

quantification of β -hydroxytetradecanoic acid) and free CoA were available as authentic standards and were quantified *via* an eight-point calibration curve ranging from 25 μ M to 1 mM. Other analytes were identified by exact mass and their concentrations were estimated using the most chemically similar compounds for which authentic standards were available.

2.6. Analyzing methyl ketones and fatty acids from cell cultures by gas chromatography-mass spectrometry (GC-MS)

For methyl ketone production, frozen glycerol stocks of *E. coli* strains were used as inocula and grown in 50 mL of TSB or M9-MOPS minimal medium (Zhang et al., 2012b) in 250-mL flasks with 200-rpm agitation at 37°C for up to 96 hours. Final concentrations of 0.1 to 0.5 mM of IPTG and 1 mM of arabinose (unless indicated otherwise) were added to cultures after 6 hours to induce gene expression. Five milliliters of decane (Sigma-Aldrich, ReagentPlus $\geq$ 99% purity) or dodecane (for RAMOS experiments, Sigma-Aldrich, ReagentPlus $\geq$ 99% purity), amended with perdeuterated tetracosane ($C_{24}D_{50}$) and 3-tetradecanone (Sigma-Aldrich) as internal standards, were added to the cultures during induction. To measure methyl ketones and fatty acids from cultures, 50 μ L of the decane overlay was removed at selected time points for analysis by electron ionization (EI) GC-MS with a quadrupole mass spectrometer as previously described (Goh et al., 2012).

2.7. Measuring glucose utilization and metabolite production during growth by high-performance liquid chromatography (HPLC)

Glucose and organic acids from cell cultures were measured by an Agilent 1100 Series HPLC system equipped with an Agilent 1200 Series refractive index detector (RID) (Agilent Technologies, CA) and Aminex HPX-87H ion-exclusion column (300-mm length, 7.8-mm internal diameter; Bio-Rad Laboratories, Inc., Hercules, CA). One hundred-microliter aliquots of

cell cultures were removed at various time points during production and filtered through a spin-cartridge with a 0.45- μ m nylon membrane, and 5 μ L of the filtrate was eluted through the column at 50°C with 4 mM sulfuric acid at a flow rate of 600 μ L/min for 25 min. Metabolites were quantified by using external standard calibration with authentic standards.

2.8. Fed-batch fermentation for methyl ketone production

Fed-batch fermentation was carried out in a 2-L bioreactor equipped with a Sartorius BIOSTAT[®] B plus control unit for regulating dissolved oxygen (DO), pH, and temperature. A 20-mL overnight culture prepared from a frozen glycerol stock was cultured in a 250-mL flask as previously described. This culture was used to inoculate 710 mL of medium in the bioreactor. The medium for batch phase was composed of M9 salts supplemented with 0.3 g/L of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 5 g/L of NH_4Cl , 5 mg/L thiamine, 5.56 mg/L of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 44 mg/L $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, micronutrients [consisting of 4 mM $(\text{NH}_4)_6\text{Mo}_7\text{O}_{24}$, 0.53 mM boric acid, 40 mM CoCl_2 , 20 mM CuSO_4 , 106 mM MnCl_2 , and 13 mM ZnSO_4], 22 g/L of glucose, and appropriate antibiotics. One hundred and fifty milliliters of dodecane overlay amended with 0.5 g/L of 3-tetradecanone as an internal standard was also added to the bioreactor at the beginning of batch phase. The culture was maintained at pH 6.5 automatically by the addition of a base solution (7 M NH_4OH and 5 M KOH). The temperature was maintained at 37°C and airflow was sustained at a rate of 0.1 VVM (volume of air per volume of liquid per minute) throughout the fermentation run. Dissolved oxygen was maintained at 22% *via* regulated stirrer speed. Cultures were induced with 1 mM arabinose and 0.1 mM IPTG at 7 hours after initiation of batch phase. Batch phase was carried out until glucose was depleted (~22.8 hrs), at which time glucose feeding was initiated. The feed solution contained approximately 505 g/L glucose, 15 g/L $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, micronutrients (at 3X concentration of the batch media) and appropriate

antibiotics. The initial feed rate was set at 0.056 ml/min. The feeding strategy employed an automatic algorithm to create feed pulses by increasing the feed rate by 26% of the initial rate for 2.5 minutes. Following this pulse, if the DO was more than 24%, feed rate was increased by 7%, otherwise the new feed rate was decreased by 7%. The feed rate was kept constant for 60 min until the next pulse started. This cycle was repeated until all feed solution (ca. 577 mL) had been added to the fermenter.

3. Results and Discussion

3.1. Decarboxylation of β -keto acids to form methyl ketones occurs spontaneously

Unlike the pathway that we have developed, other recently reported heterologous pathways for methyl ketone production include a decarboxylase to catalyze the final step, namely, decarboxylation of a β -keto acid to form a methyl ketone (Park et al., 2012; Lan et al., 2013). Previously, we hypothesized that the last (decarboxylation) step of our methyl ketone pathway occurs spontaneously (Goh et al., 2012). This was supported by *in vitro* data, in which assays containing only palmitoleyl-CoA [(Z)-9-hexadecenoyl-CoA], ACO, FadB, and required cofactors were sufficient to generate a compound tentatively identified as (Z)-8-pentadecen-2-one (15:1 methyl ketone) (Goh et al., 2012). To more definitively confirm our hypothesis, we carried out *in vitro* assays with myristoyl-CoA (14:0), all three purified pathway enzymes, and required cofactors and showed that, within an hour, the corresponding methyl ketone, 2-tridecanone, was detected by LC-APCI-TOF (Figure 1A). The peak identified as 2-tridecanone had the same retention time and mass-to-charge ratio (m/z) of the $[M+H]^+$ ion (within 1.6 ppm) as an authentic 2-tridecanone standard. Furthermore, we observed near-quantitative conversion of the C₁₄ β -keto acid to the C₁₃ methyl ketone (2-tridecanone) (Figure 1B), demonstrating that ACO, FadB, and FadM are sufficient to produce methyl ketones from acyl-CoAs without any

decarboxylase; this assumes that ACO, FadB, and/or FadM do not have any unreported decarboxylase activity. Although an authentic standard was not available for the C₁₄ β-keto acid (3-oxotetradecanoic acid), there were two key lines of evidence that strongly support its identification. First, in the LC-APCI TOF data (Figure 1A), the putative C₁₄ β-keto acid peak eluted much earlier than the C₁₃ methyl ketone, as would be expected for a reverse-phase separation of a carboxylic acid from a methyl ketone, and the m/z ratio of its [M+H]⁺ ion was virtually identical to that of the confirmed C₁₃ methyl ketone (within 3.5 ppm). Thus, it appears that the C₁₄ β-keto acid was chromatographically separated from the C₁₃ methyl ketone but was decarboxylated to form a methyl ketone when it entered the APCI ion source. Second, LC-ESI-TOF analysis detected a peak with an m/z value of 241.1826 for the [M-H]⁻ ion, which is within 6.6 ppm of the expected m/z value for a C₁₄ β-keto acid (Figure S1). The ESI ion source is lower in energy than the APCI source and thus did not induce instantaneous decarboxylation.

3.2. *In vitro* assays show that the FadM thioesterase is promiscuous

In addition to confirming that decarboxylation of β-keto acids occurs spontaneously, *in vitro* assays suggested that the FadM thioesterase also acts on CoA-thioester metabolites other than β-ketoacyl-CoAs. This became apparent when free acid versions of the CoA-thioester intermediates were detected when the methyl ketone pathway was allowed to progress to various steps in the presence of FadM (the pathway is shown in Figure 2A). For example, incubation of 14:0 acyl-CoA (myristoyl-CoA) with FadM resulted in complete hydrolysis into myristic acid within one hour (Figure 2B). In addition, 14:1 free acid was detected when ACO, FAD, and FadM were present in the assay, although the rate of hydrolysis of the 14:1 acyl-CoA appears to have been slower than that of the 14:0 acyl-CoA (i.e., the ratio of the 14:1 free acid to the CoA thioester was relatively low after 1 hr) (Figure 2B). (When such experiments were conducted

both in the presence and absence of FadM, the 14:1 free acid was only detected in the presence of FadM, indicating that FadM was responsible for the hydrolysis (data not shown.) Further, when myristoyl-CoA was incubated with ACO, FAD, FadB, and FadM to generate β -hydroxymyristoyl-CoA, there was complete hydrolysis to the β -hydroxy acid within 1 hr. Finally, when myristoyl-CoA was incubated with ACO, FAD, FadB, NAD^+ , and FadM to generate β -ketomyristoyl-CoA, only the β -keto acid and no β -ketoacyl-CoA was detected within 1 hr. The relatively broad substrate range of FadM is not surprising, as this enzyme was previously shown to exhibit thioesterase activity toward a wide range of medium- and long-chain CoA thioester intermediates from the β -oxidation pathway (Nie et al., 2008). However, the relatively high apparent thioesterase activity of FadM on β -ketomyristoyl-CoA is interesting, and this compound was not tested by Nie et al. (2008).

Our *in vitro* data suggest that tuning the expression of FadM relative to the rest of the pathway enzymes may be important for maximizing flux to β -ketoacyl-CoAs by minimizing the diversion of CoA-thioester intermediates to dead-end, free acid products. However, we should point out that concentrations of these dead-end, free-acid products were negligible during *in vivo* production (data not shown). Possible explanations for this observation are that: (1) *in vitro* and *in vivo* conditions are different, including the absolute and relative concentrations of pathway proteins, (2) the promiscuous activity of FadM could be masked *in vivo* by re-formation of CoA thioesters catalyzed by FadD, and (3) flux through the methyl ketone pathway toward β -ketoacyl-CoAs may be sufficiently fast *in vivo* to preclude significant hydrolysis of CoA-thioester intermediates by FadM.

3.3. Increasing fatty acid production and flux into the β -oxidation pathway for improved methyl ketone production

In addition to *in vitro* characterization of the methyl ketone pathway, we pursued several improvements to the pathway, including metabolically engineering strains to increase flux through our modified version of β -oxidation. Methyl ketones derive from fatty acids (Figure 2A) and conversion of fatty acids into acyl-CoAs, catalyzed by FadD (fatty acyl-CoA synthetase), is the first committed step of β -oxidation and the gateway for entry of fatty acids into the methyl ketone pathway (Figure 2A). In our previous report, free fatty acids were still detected at the end of methyl ketone production by the best producing strain (EGS895), even though this strain exhibited lower ratios of fatty acid precursors to their daughter methyl ketones compared to lesser-producing strains (Goh et al., 2012). This suggested that there was suboptimal pull of free fatty acids into the β -oxidation pathway. An initial attempt to increase the free fatty acid flux into the methyl ketone pathway by overexpressing FadD was unsuccessful and resulted in a two-fold decrease in methyl ketone production (Goh et al., 2012).

Another strategy for increasing methyl ketone production is to increase fatty acid production by overexpressing the fatty acid-responsive transcription factor, FadR. It was recently shown that overexpressing FadR increased free fatty acid titers 7.5-fold in *E. coli* (Zhang et al., 2012b). We evaluated this strategy to enhance methyl ketone production. The *fadR* gene was cloned in three different configurations to assess which would most enhance methyl ketone titer: (1) *fadR* driven by the *trc* promoter (P_{trc}) (strain EGS1185; Table 1), (2) *fadR* cloned downstream of *fadM* to create an operon driven by the P_{trc} promoter (strain EGS1190; Table 1), and (3) *fadR* driven by the *araBAD* promoter (P_{BAD}) (strain EGS1230; Table 1). Unfortunately, all three configurations resulted in at least two-fold lower methyl ketone production than in the absence of the cloned *fadR* gene, even though they increased fatty acid titer in some cases (e.g., strain EGS1230 had 1.6-fold higher free fatty acids than strain EGS895). We speculated that

higher fatty acid titers were not translating into higher methyl ketone titers because conversion of fatty acids to acyl-CoAs was a bottleneck. To test this, we co-overexpressed *fadD* with *fadR* (resulting in strain EGS1320; Table 1) and observed a ca. 4-fold increase in methyl ketone titer (Figure 3). We also tested methyl ketone production by strain EGS1320 with varying inducer (arabinose) concentrations to modulate *fadR* and *fadD* expression levels. At all tested concentrations (0.5 to 5 mM arabinose), methyl ketone production was always lower than in the absence of arabinose, indicating that basal leaky expression of *fadR* and *fadD* was optimal for methyl ketone production. Under these conditions, negligible amounts of free fatty acids were detected in strain EGS1320 during production, suggesting that we had created a more efficient metabolic pull of free fatty acids into the β -oxidation pathway (Figure S3).

We also attempted to use another strategy to increase the free fatty acid pool: enhancing malonyl-CoA formation *via* acetyl-CoA carboxylase (ACC) overexpression. Davis et al. (2000) observed a 6-fold enhancement in fatty acid titer in *E. coli* by overexpressing the native ACC (consisting of four subunits: AccA, AccB, AccC, and AccD). However, they found that the ratios of all four ACC subunits had to be balanced to avoid toxicity effects (Davis et al., 2000). More recently, overexpression of the ACC complex from *Corynebacterium glutamicum*, which consists of only two subunits (encoded by *accBC* and *dtsRI*), was engineered into *E. coli* to alleviate the bottleneck of malonyl-CoA for increased (2*S*)-flavanone production (Miyahisa et al., 2005). Zha and co-workers subsequently confirmed that the overexpression of ACC from *C. glutamicum* resulted in three-fold greater malonyl-CoA in *E. coli* (Zha et al., 2009). In light of these data, we proceeded with overexpressing the ACC complex from *C. glutamicum* in our engineered methyl ketone-producing strain (strain EGS1220, Table 1). Overexpressing the heterologous ACC complex improved free fatty acid titers 4-fold but decreased methyl ketone

production by almost ten-fold. We also cloned the ACC complex into strain EGS1320 (which resulted in strain EGS1565, Table 1) but observed a two-fold decrease in methyl ketone production relative to its parent strain. Since our attempts at overexpressing the ACC complex tended to improve fatty acid titers but decrease methyl ketone titers, even in strains overexpressing codon-optimized *fadD*, we did not pursue this strategy further.

3.4. Consolidating methyl ketone pathway enzymes onto one plasmid

The methyl ketone pathway genes were originally borne on two plasmids (Figure 4A; Table 1). We decided to consolidate the pathway genes onto a single plasmid so that one less antibiotic would be needed to retain the plasmids through selection pressure. Our initial one-plasmid strain, EGS1640, was obtained by sub-cloning the promoters and gene fragments from both plasmids into the BglBrick vector, pBbE8k-RFP, which contained the *colE1* origin of replication (Lee et al., 2011). However, strain EGS1640 produced 14-fold lower methyl ketone titers than the corresponding two-plasmid strain, EGS1370 (Figure S4). Targeted proteomics analysis revealed that the acyl-CoA oxidase protein was very poorly expressed in EGS1640. Since the *aco* gene (Mlut_11700) was cloned from the Gram-positive actinobacterium, *Micrococcus luteus*, whose genome has a GC content of ~ 73% (Young et al., 2010), we codon-optimized the gene for expression in *E. coli*. Replacing the original *aco* gene with a codon-optimized version resulted in EGS1710 (Figure 4B), which not only had dramatically better ACO expression than strain EGS1640 (Figure S4), but also had better (~ 45%) methyl ketone production than strain EGS1370 (Figure 3).

After the pathway genes were reconfigured into a single plasmid for strain EGS1710, we considered that the ratios of the expressed pathway enzymes may have changed and again tested a range of inducer (IPTG and arabinose) concentrations. Indeed, we observed that optimal

inducer concentrations had changed to 0.1 mM IPTG and 1 mM arabinose (recall that no arabinose was the optimal condition for methyl ketone production in strain EGS1320).

3.5. Engineering of the *E. coli* host to reduce acetate production

Production of methyl ketones was initially performed in TSB complex medium (which contains 0.2% glucose) (Goh et al. 2012). However, using complex or rich media such as TSB or EZ-Rich would not be economically viable for large-scale production. The transition to use of M9-MOPS minimal medium resulted in significant accumulation of acetate. Specifically, in 1% and 2% glucose, up to 2 g/L and 6 g/L of acetate was detected, respectively. Acetate accumulation during growth is undesirable because it is known to inhibit protein expression even at relatively low concentrations (e.g., 0.5 g/L) and also diverts carbon flux away from formation of the desired products (Eiteman and Altman, 2006). Acetate production and excretion in *E. coli* have been well characterized and occur *via* two major pathways under aerobic conditions (Wolfe, 2005). In one pathway, pyruvate oxidase (PoxB) converts pyruvate to acetate, while in another, phosphotransacetylase (Pta) converts acetyl-CoA to acetyl phosphate, and subsequently, acetate kinase (AckA) dephosphorylates acetyl phosphate to acetate. We created chromosomal knockouts of each of these pathways (ESG1710 with $\Delta poxB$; EGS1890 with $\Delta ackA-ptA$) as well as the triple knockout (EGS1895 with $\Delta poxB$ and $\Delta ackA-ptA$). Initially, we compared the two-plasmid strain in either the $poxB^+$ or $poxB^-$ background (strain EGS1320 versus EGS1370, respectively), and observed an 11-fold improvement in methyl ketone production in the $poxB^-$ strain compared to the $poxB^+$ strain (Figure 3). Subsequently, we compared the one-plasmid pathway in the various acetate-knockout hosts and observed that methyl ketone production in the $\Delta ackA-ptA$ background (strains EGS1890 and 1895) was about 2.5-fold greater than in the $\Delta poxB$ background (strain EGS1710) (Figure 3). Measurement of acetate at the end of production

showed none detectable in 1% glucose and < 3 g/L in 2% glucose. Thus, knocking out the major acetate pathways not only lowered acetate accumulation but also improved methyl ketone production. The triple-knockout strain, EGS1895, is currently our best producer. In 1% glucose (M9-MOPS minimal medium), it is capable of producing ~ 1.4 g/L of methyl ketones (Figure 3), which represents 40% of the maximum theoretical yield.

3.6. Allocation of glucose carbon for the best methyl ketone producers

To better understand glucose metabolism in our three best methyl ketone-producing strains (EGS1710, EGS1890, and EGS1895), we characterized glucose consumption and production of methyl ketones, CO₂, biomass, and acetate in these strains in the presence of 1% and 2% glucose over 90 hours (Figure 5). Noteworthy trends apparent in these data include the following: (a) methyl ketone production was greater and glucose depletion more rapid in $\Delta ackA$ -*pta* knockout strains (EGS1890 and EGS1895) compared to the $\Delta poxB$ strain (EGS1710) and (b) low levels of acetate (never exceeding 1 g/L and 2 g/L acetate for 1% and 2% glucose, respectively) were released as glucose was consumed, and the acetate was re-assimilated (presumably *via* AMP-forming acetyl-CoA synthetase (Wolfe, 2005)) upon complete depletion of glucose. Interestingly, there was still acetate production in the triple acetate knockout strain (especially with 2% glucose). In fact, acetate production was slightly higher in the $\Delta ackA$ -*pta* strains than in the $\Delta poxB$ strain (maximum of ~1.7 g/L versus ~1.2 g/L, respectively), indicating that acetate was being produced *via* an alternative pathway. One possible acetate source is from the reaction of acetoacetyl-CoA transferase (encoded by *atoA* and *atoD*), whereby a fatty acid and acetyl-CoA, which may be at higher concentrations in our engineered strains than for the wild type, are converted to an acyl-CoA and acetate. The rate of acetate re-assimilation was also different among the knockout strains. In the $\Delta poxB$ strain (EGS1710), acetate was rapidly re-

assimilated as soon as glucose consumption was complete. In contrast, in the $\Delta ackA$ -pta strains (EGS1890 and EGS1895), acetate concentrations decreased slowly after glucose depletion but acetate was never fully re-assimilated. This suggests that the $\Delta poxB$ strain is better configured to overcome the metabolic burden of assimilating acetate (1 ATP is required for the AMP-forming acetyl-CoA synthetase) than the $\Delta ackA$ -pta strains. A potentially related observation is that the $\Delta poxB$ strain (EGS1710) directed more glucose carbon to biomass after 90 hr of incubation than did the $\Delta ackA$ -pta strains (Table 2). Methyl ketone yields were generally comparable for strains EGS1710, EGS1890, and EGS1895 in 1% and 2% glucose (Table 2), as were distributions of individual methyl ketones (Table S4). Overall, loss of carbon to CO₂ was substantial in all three best strains (41-53% of glucose carbon; Table 2). A relatively small portion of this CO₂ loss can be attributed to methyl ketone biosynthesis; based on the amount of methyl ketones produced and accounting for decarboxylation reactions (pyruvate dehydrogenase, FabH and FabF, and spontaneous β -keto acid decarboxylation) and carboxylation reactions (acetyl-CoA carboxylase) in fatty acid and methyl ketone biosynthesis *via* our engineered pathway, approximately 3-8% of glucose carbon was released as CO₂ attributable to our pathway. Thus, although we have improved methyl ketone titers in minimal medium more than 160-fold relative to our previously reported best strain (Figure 3), there is still carbon flux to CO₂ and biomass that could be more efficiently diverted to methyl ketone production.

3.7. Fed-batch fermentation with best strain (EGS1895)

Fed-batch fermentation of strain EGS1895 demonstrated nearly linear methyl ketone production over time while glucose was being fed, from approximately 23 to 45 hr, and methyl ketone production corresponded well with glucose consumption (Figure 6). The maximum yield during fermentation was 30% of the average value observed during batch production in shake

flasks (0.042 g MK/g glucose, Figure 6, versus 0.14 g MK/g glucose, Figure 3). Acetate and pyruvate concentrations remained relatively low in batch mode (2% glucose) but rose to 4.8 and 4.4 g/L, respectively, by 45 hr.

4. Conclusions

We have used *in vitro* studies to gain insights into the recently developed pathway (Goh et al. 2012) that produces C₁₁ to C₁₇ methyl ketones in *E. coli*. In particular, these studies (1) confirmed that a decarboxylase is not required to convert β -keto acids into methyl ketones (this reaction occurs spontaneously) and (2) showed that FadM is promiscuous and can hydrolyze not only β -ketoacyl-CoAs but also several CoA-thioester pathway intermediates, potentially decreasing flux through the pathway by creating dead-end, free-acid products. However, these dead-end products were not found to have accumulated in the growth medium during *in vivo* production studies.

We have also made pathway and host engineering modifications to our best previously reported methyl ketone-producing *E. coli* strain (Goh et al. 2012), resulting in a 160-fold titer improvement in minimal medium. The best strain in the present study attained a methyl ketone titer of 1.4 g/L with 1% glucose in minimal medium, representing 40% of the maximum theoretical yield. This strain reached a titer of 3.4 g/L after ~45 hr of fed-batch fermentation with glucose. Engineering improvements included increasing production of free fatty acids and their flux into the methyl ketone pathway by balancing overexpression of the genes encoding the fatty acid-responsive transcription factor, *fadR*, and fatty acyl-CoA synthetase, *fadD*. Multiple parameters for *fadR* and *fadD* expression were tested individually and in combination (P_{BAD} and P_{trc} , a range of inducer concentrations, various operon configurations, and codon optimization for *fadD*), and the resulting methyl ketone titers were sensitive to these parameters. Attempts to

increase fatty acid, and ultimately, methyl ketone production by overexpressing a heterologous acetyl-CoA carboxylase resulted in increases in fatty acid production but sharp decreases in methyl ketone production. Improvements in methyl ketone titer also resulted from consolidation of the pathway on a single plasmid, codon optimization of the acyl-CoA oxidase from *Micrococcus luteus*, and knockouts of key acetate production pathways (*poxB*, *ackA*, and *pta*). Despite the large improvements in methyl ketone titer and yield, carbon mass balance studies with 1 or 2% glucose demonstrated that there is still carbon flux to CO₂ (41 to 53% of glucose carbon) and biomass that could be more efficiently diverted to methyl ketone production. In the future, we will leverage the use of metabolic flux analysis to determine which additional modifications will most effectively improve our methyl ketone yields and titers.

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Appendix A. Supplementary material

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528

FIGURE LEGENDS

Figure 1. Mass spectrometric data from *in vitro* assays demonstrating spontaneous (enzyme-free) decarboxylation of β -keto acids to form methyl ketones. (A) LC-APCI-TOF chromatogram of (top) *in vitro* assay initially containing tetradecanoyl-CoA, ACO, FadB, FadM, NAD⁺, and FAD and incubated for 45 min and (bottom) 2-tridecanone authentic standard. (B) Time course of *in vitro* assay showing concentrations of β -ketotetradecanoic acid (blue) and 2-tridecanone (purple).

Figure 2. (A) Re-engineered β -oxidation pathway designed for methyl ketone production. Black boxes represent overexpressed enzymes and gray circles represent genes that were chromosomally deleted (see Goh et al., 2012 and text). The dashed arrows represent potential side reactions catalyzed by the FadM thioesterase. (B) *In vitro* assay results showing concentrations (determined by LC-ESI-TOF and LC-APCI-TOF mass spectrometry) of CoA-thioester and β -keto acid intermediates, free acid dead-end products, and methyl ketones at T = 0 and 1 hour. Assay conditions allowing the reactions to progress through various pathway steps are shown on the vertical axis. Approximately 1 mM of myristoyl-CoA was added to the assays, so it is clear that the detected metabolites typically accounted for more than 80 mol% of this substrate. A table containing the averages and standard deviations for the data represented in Figure 2B are provided in Table S3. In addition, mass balance with respect to CoA (i.e., CoA-thioester intermediates and free CoA) is presented in Figure S2.

Figure 3. History of strain configuration and corresponding titer in the development of a methyl ketone pathway in *E. coli* based on re-engineered β -oxidation. Bar heights represent averages ($n \geq 6$) and error bars represent 1 standard deviation of titers determined after 72 hr of incubation; total methyl ketone titers in mg/L are indicated above the bars. "co-aco" represents a codon-optimized version of acyl-CoA oxidase from *Micrococcus luteus*. Gray bars represent titers for strains grown in TSB (0.2% glucose) and reported by Goh et al. (2012) and black bars represent strains grown in M9-MOPS minimal medium (1% glucose).

Figure 4. Plasmid maps of the (A) two-plasmid system used in strains EGS1320 and EGS1370 and (B) the one-plasmid system used in strains EGS1710, EGS1890, and EGS1895.

Figure 5. Time-course data for the three best producing strains in this study: EGS1710 ($\Delta poxB$), EGS1890 ($\Delta ackA-ptd$), and EGS1895 ($\Delta ackA-ptd, \Delta poxB$). Symbols: methyl ketones (red triangles), glucose (blue diamonds), acetate (green squares), OD₆₀₀ (black circles), 1% glucose (solid lines with filled symbols), and 2% glucose (dotted lines with open symbols). Analytes not detected included lactate, pyruvate, formate, and ethanol.

Figure 6. Fed-batch fermentation of strain EGS1895 with glucose. The initial batch phase with 2% glucose ended at ~23 hr, at which time glucose feeding commenced.

571 TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant Characteristics	Source or reference
<i>E. coli</i> strains		
BL21 (DE3)	F ⁻ <i>ompT gal dcm lon hsdSB</i> (r _B ⁻ m _B ⁻) λ(DE3)	(Studier and Moffatt, 1986)
DH1	<i>endA1 recA1 gyrA96 thi-1 glnV44 relA1 hsdR17</i> (r _K ⁻ m _K ⁺) λ ⁻	(Meselson and Yuan, 1968)
LT-□ <i>fadE</i>	DH1 Δ <i>fadE</i> with pKS1	(Steen et al., 2010)
EGS084	LT-□ <i>fadE</i> with pEC-XK99E	(Beller et al., 2010)
EGS514	BL21(DE3) with pEG513	(Goh et al. 2012)
EGS517	BL21(DE3) with pEG516	(Goh et al. 2012)
EGS522	DH1; Δ <i>fadE</i> ; Δ <i>fadA</i>	(Goh et al. 2012)
EGS560	EGS522 with pEG530 and pEC-XK99E	(Goh et al. 2012)
EGS860	LT-Δ <i>fadE</i> with pEG855	(Goh et al. 2012)
EGS895	EGS522 with pEG530 and pEG855	(Goh et al. 2012)
EGS1127	BL21(DE3) with pEG1125	This study
EGS1185	EGS522 with pEG530 and pEG1165	This study
EGS1190	EGS522 with pEG530 and pEG1170	This study
EGS1220	EGS522 with pEG530 and pEG1200	This study
EGS1230	EGS522 with pEG530 and pEG1210	This study
EGS1320	EGS522 with pEG530 and pEG1315	This study
EGS1350	DH1; Δ <i>fadE</i> ; Δ <i>fadA</i> ; Δ <i>poxB</i>	This study
EGS1370	EGS1350 with pEG530 and pEG1315	This study
EGS1400	DH1; Δ <i>fadE</i> ; Δ <i>fadA</i> ; Δ <i>ackA-pta</i>	This study
EGS1405	DH1; Δ <i>fadE</i> ; Δ <i>fadA</i> ; Δ <i>ackA-pta</i> ; Δ <i>poxB</i>	This study
EGS1565	EGS522 with pEG530 and pEG1545	This study
EGS1640	EGS1350 with pEG1610	This study
EGS1710	EGS1350 with pEG1675	This study
EGS1890	EGS1400 with pEG1675	This study
EGS1895	EGS1405 with pEG1675	This study
Plasmids		
pBbA5c-RFP	Cm ^r ; p15a derivative BglBrick vector containing RFP under the <i>lacUV5</i> promoter (P _{lacUV5}).	(Lee et al., 2011)
pBbE8k-RFP	Km ^r ; ColE1 derivative BglBrick vector containing RFP under the <i>araBAD</i> promoter (P _{BAD}).	(Lee et al., 2011)
pE8a-fadR	Amp ^r ; ColE1 derivative containing <i>fadR</i> under the <i>araBAD</i> promoter (P _{BAD}).	(Zhang et al., 2012b)
pEC-XK99E	Km ^r ; <i>E. coli</i> - <i>C. glutamicum</i> shuttle expression vectors based on the medium copy number plasmid pGA1 and containing the <i>trc</i> promoter (P _{trc})	(Kirchner and Tauch, 2003)
pKS1	Cm ^r ; p15a derivative containing ' <i>tesA</i> under P _{lacUV5}	(Steen et al., 2010)
pEG513	Km ^r ; ~2.2-kb PCR fragment of <i>fadB</i> cloned into pSKB3 at <i>NdeI</i> and <i>SalI</i> sites.	(Goh et al. 2012)
pEG516	Km ^r ; ~2.1-kb fragment of Mlut_11700 cloned into	(Goh et al. 2012)

pEG530	pSKB3 at <i>NdeI</i> and <i>SalI</i> sites. Cm ^r ; ~2.1-kb PCR fragment of Mlut_11700 and ~2.2-kb PCR fragment of <i>fadB</i> cloned downstream of the ' <i>tesA</i> gene in pKS1 by SLIC.	(Goh et al. 2012)
pEG855	Km ^r ; ~0.4-kb PCR fragment of <i>fadM</i> cloned into pEC-XK99E at <i>EcoRI</i> and <i>XbaI</i> sites.	(Goh et al. 2012)
pEG1125	Km ^r ; ~0.4-kb PCR fragment of <i>fadM</i> cloned into pSKB3 at <i>NdeI</i> and <i>SalI</i> sites.	This study
pEG1165	Km ^r ; ~0.25-kb PCR fragment of P _{trc} and ~0.72-kb PCR fragment of <i>fadR</i> cloned into <i>SalI</i> site of pEG855 by SLIC.	This study
pEG1170	Km ^r ; ~0.72-kb PCR fragment of <i>fadR</i> cloned into <i>SalI</i> site downstream of <i>fadM</i> in pEG855 by SLIC.	This study
pEG1200	Km ^r ; ~2.0-kb PCR fragment of P _{trc} - <i>accBC</i> and ~1.9-kb PCR fragment of P _{trc} - <i>dtsR1</i> cloned into <i>SalI/XbaI</i> sites of pEG855 by SLIC.	This study
pEG1210	Km ^r ; ~2.2-kb PCR fragment of <i>araC</i> -P _{BAD} - <i>fadR</i> of pE8a- <i>fadR</i> cloned into <i>NdeI</i> site of pEG855 by SLIC.	This study
pEG1315	Km ^r ; ~1.7-kb PCR fragment of codon optimized <i>fadD</i> (see Table S2) cloned into <i>BamHI</i> site of pEG1210 by SLIC.	This study
pEG1545	Km ^r ; ~4.0-kb PCR fragment of P _{trc} - <i>accBC</i> -P _{trc} - <i>dtsR1</i> from pEG1200 cloned into <i>XbaI</i> site of pEG1315 by SLIC.	This study
pEG1610	Km ^r , ~6.6-kb fragment of <i>araC</i> -P _{BAD} - <i>fadR</i> -co- <i>fadD</i> -P _{trc} - <i>fadM</i> fragment from pEG1315 and ~5.1-kb fragment of P _{lacUV5} -' <i>tesA</i> - <i>fadB</i> - <i>aco</i> from pEG530 cloned into pBbE8k-RFP at the <i>BamHI</i> and <i>AatII</i> sites.	This study
pEG1615	Cm ^r , same as pEG530 except Mlut_11700 was replaced with a version that is codon-optimized for expression in <i>E. coli</i> (see Table S2) using <i>BamHI</i> sites.	This study
pEG1675	Km ^r , ~6.6-kb fragment of <i>araC</i> -P _{BAD} - <i>fadR</i> -co- <i>fadD</i> -P _{trc} - <i>fadM</i> fragment from pEG1315 and ~5.1-kb fragment of P _{lacUV5} -' <i>tesA</i> - <i>fadB</i> -co- <i>aco</i> from pEG1615 cloned into pBbE8k-RFP at the <i>BamHI</i> and <i>AatII</i> sites.	This study
pSKB3	Km ^r ; A derivative of the expression vector pET-28a with the thrombin protease site replaced by a TEV ^b protease site.	Burley ^c

^a Codon-optimized *fadD* gene was obtained from Fuzhong Zhang.

^b TEV, tobacco etch virus

^c Stephen K. Burley

Table 2. Carbon mass balance in three best methyl ketone-producing strains^a

	Strains	Methyl ketones^b	Biomass^{b,c}	CO₂^b	Acetate^b	Total^b
1% glucose	EGS1710	7.6	41	42	0.0	90
	EGS1890	17	27	53	0.0	97
	EGS1895	19	25	52	0.0	95
2% glucose	EGS1710	9.4	35	41	0.0	86
	EGS1890	14	26	42	4.0	86
	EGS1895	16	24	47	3.0	90

^a Data are from the same experiment represented in Figure 5.

^b Expressed as % of glucose carbon.

^c Assuming that 50% of cell dry weight is carbon (Moran et al., 2010)

Figure 1

Figure 2

Methyl ketones (mg/L)

Legend:
 ■ TSB
 ■ M9-MOPS (1% Gluc)

Goh et al. 2012

Strain	Media	Methyl ketones (mg/L)	Genetic Background
EGS084	TSB	0.12	<i>tesA</i> , Δ <i>fadE</i>
EGS860	TSB	0.62	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i>
EGS560	TSB	10	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i>
EGS895	TSB	120	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i>
EGS895	M9-MOPS (1% Gluc)	8.6	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i>
EGS1320	M9-MOPS (1% Gluc)	36	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i> , 1-plasmid, <i>co-aco</i>
EGS1370	M9-MOPS (1% Gluc)	390	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i> , 1-plasmid, <i>co-aco</i> , Δ <i>ackA-pta</i>
EGS1710	M9-MOPS (1% Gluc)	570	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i> , 1-plasmid, <i>co-aco</i> , Δ <i>ackA-pta</i>
EGS1890	M9-MOPS (1% Gluc)	1350	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i> , 1-plasmid, <i>co-aco</i> , Δ <i>ackA-pta</i>
EGS1895	M9-MOPS (1% Gluc)	1400	<i>tesA</i> , Δ <i>fadE</i> , <i>fadM</i> , <i>fadB</i> , Δ <i>fadA</i> , <i>fadR</i> , <i>fadD</i> , Δ <i>poxB</i> , 1-plasmid, <i>co-aco</i> , Δ <i>ackA-pta</i>

Figure 4

Figure 5

Figure 6