Fermentative production of short-chain fatty acids in *Escherichia coli*

Alexandra R. Volker, David S. Gogerty,† Christian Bartholomay,‡ Tracie Hennen-Bierwagen, Huilin Zhu§ and Thomas A. Bobik

Roy J. Carver Department of Biochemistry, Biophysics and Molecular Biology Iowa State University, Ames, IA 50011, USA

*Escherichia coli* was engineered for the production of even- and odd-chain fatty acids (FAs) by fermentation. Co-production of thiolase, hydroxybutyryl-CoA dehydrogenase, crotonase and trans-enoyl-CoA reductase from a synthetic operon allowed the production of butyrate, hexanoate and octanoate. Elimination of native fermentation pathways by genetic deletion (ΔdhA, ΔadhE, ΔackA, Δpta, ΔfrdC) helped eliminate undesired by-products and increase product yields. Initial butyrate production rates were high (0.7 g l⁻¹ h⁻¹) but quickly levelled off and further study suggested this was due to product toxicity and/or acidification of the growth medium. Results also showed that endogenous thioesterases significantly influenced product formation. In particular, deletion of the *yciA* thioesterase gene substantially increased hexanoate production while decreasing the production of butyrate. *E. coli* was also engineered to co-produce enzymes for even-chain FA production (described above) together with a coenzyme B₁₂-dependent pathway for the production of propionyl-CoA, which allowed the production of odd-chain FAs (pentanoate and heptanoate). The B₁₂-dependent pathway used here has the potential to allow the production of odd-chain FAs from a single growth substrate (glucose) in a more energy-efficient manner than the prior methods.

INTRODUCTION

Fatty acids (FAs) of various chain lengths are important industrial compounds. They are used by the food and beverage and pharmaceutical industries, and can be converted chemically or enzymically into important biofuels including FA methyl esters, fatty alcohols, methyl ketones, alkenes and alkanes (Dellomonaco et al., 2010; Peralta-Yahya et al., 2012; Zhang et al., 2009). Current methods for industrial FA production often rely on petroleum, a finite resource, whose extraction and usage create energy security and environmental concerns. Hence, bio-based routes of FA production are of interest (Dellomonaco et al., 2010; Peralta-Yahya et al., 2012; Zhang et al., 2009).

Research on the bio-based production of FAs has been mostly conducted in *Escherichia coli* and *Clostridium*. The three main approaches that have been used are diversion of phospholipid biosynthesis, reverse β-oxidation and fermentation (Clomburg et al., 2012; Dellomonaco et al., 2010, 2011; Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012; Steen et al., 2010; Zhang et al., 2009). When specific thioesterases are produced at high levels in *E. coli* they hydrolyse the fatty acyl-carrier protein (ACP) intermediates of phospholipid biosynthesis and release free FAs (Lennen & Pfleger, 2012; Lu et al., 2008; Peralta-Yahya et al., 2012; Zhang et al., 2011). Diverting precursors of phospholipid biosynthesis in this manner has been used to produce FAs from C8 to C18 (Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012). The chain lengths released depend on the specificity of the thioesterase, and mixtures of FA or in some cases single FAs are produced. Reverse β-oxidation has also been used to produce FAs in *E. coli* (Clomburg et al., 2012; Dellomonaco et al., 2011). Normally, β-oxidation is used for the degradation of FAs as carbon and energy sources, but *E. coli* has been engineered to reverse this pathway for the production of FAs of various chain lengths (Clomburg et al., 2012; Dellomonaco et al., 2011). Reverse β-oxidation is potentially more efficient than redirection of phospholipid biosynthesis in the manner described above.
biosynthesis because FAs are produced by condensation of acetyl-CoA rather than malonyl-ACP whose synthesis requires additional ATP (Dellomonaco et al., 2011).

FAs have also been produced by microbial fermentation. Certain bacteria have native fermentation pathways that produce butyrate and hexanoate and have been used for the production of these FAs. Clostridium tyrobutyricum was used to produce high levels of butyric acid from various substrates in a fibrous bed reactor (Jiang et al., 2010; Wei et al., 2013). Clostridium sp. BS-1 and Megasphaera elsdenii were used to produce hexanoic acid from d-galactitol and sucrose, respectively (Choi et al., 2013; Jeon et al., 2010). In addition, Clostridium acetobutylicum has been engineered for high-level butyrate production (Jang et al., 2013). However, the development of clostridia for efficient production of diverse FAs is encumbered by slow growth, a requirement for strictly anaerobic culture conditions, sporulation and relatively limited genetic tools (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). Consequently, butyrate production was engineered into E. coli (Baek et al., 2013; Lim et al., 2013; Seregina et al., 2010). The reaction sequence used was similar to the butyrate fermentation of Clostridium to the point of butyryl-CoA formation. Two acetyl-CoA are condensed to acetocacetyl-CoA, and then converted in three steps to butyryl-CoA. However, the final step was the hydrolysis of butyryl-CoA to HS-CoA and butyrate by the TesB thioesterase, which differs from the clostridial pathway. The enzymes used for butyrate production in E. coli came from diverse sources and were mainly chosen based on studies aimed at optimizing 1-butanol production, which proceeds via a similar pathway to the point of butyryl-CoA (Baek et al., 2013; Bond-Watts et al., 2011; Lim et al., 2013; Shen et al., 2011). Of particular note is the use of NADH-dependent trans-enoyl-CoA reductase (Ter) in place of a flavoprotein-dependent butyryl-CoA dehydrogenase (Bcd) for the reduction of crotonyl-CoA to butyryl-CoA (Bond-Watts et al., 2011; Shen et al., 2011). Ter enzymes were found to substantially increase the production of 1-butanol (Bond-Watts et al., 2011; Shen et al., 2011) and also work well for butyrate production (Lim et al., 2013). Besides the use of Ter, butyrate production by E. coli was substantially enhanced by genetically deleting competing native fermentation pathways (Lim et al., 2013) as was previously done for the fermentative production of ethanol (Jarboe et al., 2007) and other bio-based products (Lim et al., 2013; Shen et al., 2011; Zhu et al., 2011). In addition to butyrate production, reactions similar to those described above for the conversion of two molecules of acetyl-CoA to butyryl-CoA together with TesB have been used under aerobic conditions to produce functionalized short-chain carboxylic acids, including 3-hydroxyvalerate (3-hydroxy-pentanoate) (Tseng et al., 2010), dihydroxybutyrate (Martin et al., 2013), 3-hydroxy-4-methylvalerate (Martin et al., 2013) and a variety of alcohols (Tseng & Prather, 2012). Similar to reverse β-oxidation, fermentative pathways proceed by sequential condensation of acetyl-CoA and are more energy efficient than the interruption of phospholipid biosynthesis.

The three general approaches to FA production described above proceed by condensation reactions that add two carbon atoms per cycle, and hence result in the production of even-chain compounds. However, each of these systems was modified to produce odd-chain-length products. This was done by providing a source of propionyl-CoA, which reacts with acetyl-CoA (or malonyl-ACP) in the first condensation step to produce odd-chain-length compounds. Propionyl-CoA was provided by addition of propionate to the medium (Dellomonaco et al., 2011) or by diversion of threonine biosynthesis (Tseng & Prather, 2012), which is advantageous because it provides a route to propionyl-CoA from glucose allowing the production of odd-chain products from a single relatively inexpensive growth substrate.

In this report, we describe the engineering of E. coli for the production of even- and odd-chain FAs by fermentation. Enzymes related to those previously used for the production of butyrate and 1-butanol are used for the production of butyrate, hexanoate and octanoate (Fig. 1). Studies also indicated that product toxicity and thioesterase specificity were critical to product yield and specificity. In addition, pentanoate and heptanoate were produced using a coenzyme B12-dependent pathway for propionyl-CoA production. This pathway has the potential to allow the production of odd-chain FAs from a single growth substrate (glucose) in a more energy-efficient manner than the prior methods used.

**METHODS**

**Micro-organisms and media.** The bacterial strains used in this study are derivatives of E. coli K-12 MG1655 (Table 1). The media used for bacterial growth included lysogeny broth (LB; Difco) M9 minimal medium and terrific broth (TB). M9 minimal medium consisted of (per litre) 64 g NaHPO4, 7H2O, 15 g KH2PO4, 2.5 g NaCl, 5.0 g NH4Cl, 0.493 g MgSO4, 7H2O, 0.0147 g CaCl2, 2H2O, 1 g yeast extract and 4 or 20 g glucose as indicated in the text. TB medium contained (per litre) 12 g tryptone, 24 g yeast extract, 100 ml 0.17 M KH2PO4, 100 ml 0.72 M K2HPO4 and 4 or 20 g l−1 glucose as indicated. Kanamycin was used at 50 mg l−1, ampicillin at 100 mg l−1, chloramphenicol at 20 mg l−1, coenzyme B12 at 200 nM and IPTG as indicated.

**Chemicals and reagents.** Antibiotics and coenzyme B12 were from Sigma Chemical Company. IPTG was from Diagnostic Chemicals Limited. Restriction enzymes and T4 DNA ligase were from New England Biolabs. Ethidium bromide, 2-mercaptoethanol and SDS were from Bio-Rad. Other chemicals were from Fisher Scientific.

**Construction of E. coli strains for the fermentative production of FAs.** Plasmids were constructed using standard methods for restriction digestion, ligation and electroporation (Sambrook & Russell, 2001) (Table 1). A ΔDE3 lysogenization kit from EMD4 Biosciences was used for site-specific integration of the T7 RNA polymerase gene under lacUVS control into E. coli strains where indicated. The enzymes chosen for the fermentative production of even-chain FAs included the Clostridium kluyveri DSM 555 acetyl-CoA C-acetyltransferase (CThA), accession number EDK35681, Clostridium beijerinckii NCIMB 8052 3-hydroxybutyryl-CoA dehydrogenase (CbHbd), accession number ABR32513, Clostridium kluyveri DSM 555 3-hydroxybutyryl-CoA dehydrogenase (CkHbd), accession number.
Table 1. Bacterial strains and plasmids used in this study

<table>
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<tr>
<th>Strain*</th>
<th>Description</th>
<th>Source</th>
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<tbody>
<tr>
<td>BL21(DE3)</td>
<td>F− ompT gal dcm lon hsdSB (rB− mB−) lacIq(lacZ)T10 lacY74 recA3 endA1 thi-1 hsdR17</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>DH5α</td>
<td>fhuA2 Δ(argF-lacZ)U169 phoA glnV44 Δ80A (lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17</td>
<td>New England Biolabs</td>
</tr>
<tr>
<td>BL21DE3 RIL</td>
<td>E. coli B F− ompT hsdS (rB− mB−) dcm + Ter− β-gal lacIq(lacZ)T10 lacY74 recA1 relA1 thi-1 hsdR17</td>
<td>Laboratory collection</td>
</tr>
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<td>BE400</td>
<td>E. coli K-12 MG1655 (wild-type)</td>
<td>Stratagene</td>
</tr>
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<td>BE1126</td>
<td>E. coli BL21DE3 RIL/pTA925-H6</td>
<td>This study</td>
</tr>
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<td>This study</td>
</tr>
<tr>
<td>BE1572</td>
<td>E. coli BL21DE3 RIL/pTA925-H6-CrkCl, canK</td>
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<td>BE1575</td>
<td>E. coli BL21DE3 RIL/pTA925-H6-EgTer+, canK</td>
<td>This study</td>
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<td>BE1578</td>
<td>E. coli K-12 MG1655 DE3</td>
<td>This study</td>
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<tr>
<td>BE1576</td>
<td>E. coli MG1655 DE3, ΔadhE::FRT, ΔdhA::FRT, ΔackA-pta::FRT, ΔfroCl::FRT, FRT/pBE522- thy-hbd-crt-ter' (CtkThl-CbHbd-CkCrt-EgTer')</td>
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<td>BE1576 ΔbgFC::FRT</td>
<td>This study</td>
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<td>BE1908</td>
<td>E. coli MG1655 DE3 ΔadhE::FRT, ΔdhA::FRT, ΔackA-pta::FRT, ΔfroCl::FRT, FRT/pBE522- pduP-pduCDE-pduGH</td>
<td>This study</td>
</tr>
<tr>
<td>BE1952</td>
<td>E. coli MG1655 DE3 ΔadhE::FRT, ΔdhA::FRT, ΔackA-pta::FRT, ΔfroCl::FRT, FRT/pBE522- pduP-pduCDE-pduGH (aldehyde dehydratase, diol dehydratase and reactivase). pBE1570 thy-hbd-crt-ter' (CtkThl-CbHbd-CkCrt-EgTer')</td>
<td>This study</td>
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*All strains are derivatives of E. coli K-12 MG1655.
harvested by centrifugation at 5000 g for 10 min using a Beckman JA-25 centrifuge and JA-10 rotor. Culture pellets (~1 g of cells) were resuspended in 3 ml of lysis buffer (50 mM HEPES, pH 7.0, 300 mM NaCl, 20 mM imidazole, 5 μM AEBSF protease inhibitor, 1 mM DTT), and broken using a French pressure cell (Thermo Scientific) at 20 000 p.s.i. (1.38 × 105 Pa). Crude cell extracts were cleared by centrifugation using a Beckman J-25 centrifuge with a JA-17 rotor at 25 000 g for 20 min. Cleared cell extracts were filtered using a Nalgene 0.45 μm syringe filter and used directly for enzyme assays. Protein concentrations were determined using the Bio-Rad Protein Assay Reagent.

**Histag protein purification.** Cell-free extracts (prepared as described above) were combined with 1 ml of Qiagen Ni-NTA Superflow and mixed on an orbital shaker for 30 min. The resin was placed in a 1 ml polystyrene column (Qiagen) and washed once each with 5 ml of buffer that contained 50 mM HEPES, pH 7.0, 300 mM NaCl and 50, 80 or 150 mM imidazole. Lastly, the histagged protein was eluted with 5 ml of 300 mM imidazole in the same buffer.

**Enzyme assays.** The Thl assay was based on previously established protocols (Hartmanis & Stadman, 1982; Sliwkowski & Hartmanis, 1984; Sliwkowski & Stadman, 1985). Briefly, activity was measured in a 1 ml assay that contained 100 mM Tris/HCl, pH 8.2, 25 mM MgCl2, 33 μM acetoacetyl-CoA (Sigma), 90 μM CoA (Sigma) and 5 μl of purified enzyme. Assays were incubated at 30 °C and absorbance at 303 nm was monitored using a Varian Cary 50 Bio UV-visible spectrophotometer. This assay measures the degradation of acetoacetyl-CoA to two molecules of acetyl-CoA. The initial rate of the Thl reaction was calculated using an extinction coefficient of 14 mM−1 cm−1 for acetoacetyl-CoA. The Hbd assay was based on a previously published protocol (Colby & Chen, 1992). The 1 ml assay contained 50 mM MOPS, pH 7.0, 1 mM DTT, 75 μM acetoacetyl-CoA (Sigma), 0.15 mM NADH (Sigma) and 5 μl of purified enzyme. Assays were carried out at 30 °C and absorbance at 340 nm was followed. The initial rate of Hbd was calculated using an extinction coefficient of 6.22 mM−1 cm−1 for NADH. The Crt assay was a modification of a prior protocol (Waterson & Hill, 1972). A 1 ml assay contained 45 mM Tris/HCl, pH 7.6, 0.0045 % BSA, 10 mM KCl, 10 mM NaCl, 2 mM MgCl2 and 53.44 μM crotonyl-CoA (Sigma), with 5 μl of purified enzyme. Assays were incubated at 30 °C and the absorbance at 230 nm was measured. A decrease in absorbance at 230 nm is due to the hydration of crotonyl-CoA to two molecules of acetyl-CoA. The initial rate of the Hbd reaction was calculated using an extinction coefficient of 6.7 mM−1 cm−1. The crotonyl-CoA reductase (CCR) assay contained 45 mM Tris/HCl, pH 7.6, 0.0045 % BSA, 10 mM KCl, 10 mM NaCl, 2 mM MgCl2, 53.44 μM crotonyl-CoA (Sigma) and 0.15 mM NADH (Sigma), with 5 μl of purified enzyme in a total volume of 1 ml. Assays were incubated at 30 °C and the absorbance at 340 nm was followed. The initial rate of CCR was calculated using an extinction coefficient of 6.22 mM−1 cm−1 for NADH.

**Fermentation conditions.** Fermentation strains were grown overnight in 50 ml of LB medium with appropriate antibiotics at 37 °C with shaking at 250 r.p.m. Cells were pelleted by centrifugation at 5000 g (using a Beckman J-25 centrifuge and JA-17 rotor) and then resuspended in 10 ml TB or minimal medium supplemented with 0.4 or 2% glucose and 200 mM coenzyme B12 (for production of odd-chain FAs). The OD600 was measured and cell suspensions were then resuspended in 10 ml of purified enzyme. Assays were incubated at 30 °C and the absorbance at 340 nm was followed. The initial rate of CCR was calculated using an extinction coefficient of 6.22 mM−1 cm−1 for NADH.

**Measurement of glucose and fermentation products.** The fermentation products present in culture media were measured by HPLC and/or GC. Cells were removed from fermentation medium by centrifugation followed by filtration through a cellulose acetate membrane (0.2 μm pore size) (Thermo Scientific). Filtered medium was analysed using a Varian ProStar HPLC system, equipped with a 325 UV monitor (210 nm), a 355 RI detector and a Bio-Rad HPX-87H column. The column was developed with 5 mM H2SO4 at 0.4 ml min−1, isocratic. Analytes were identified by retention time (and GC-MS as described below) and quantified by comparison of peak areas to a standard curve. The concentration of analytes was normalized to a cell density of OD600 of 20 (typical OD600 values were 18–22). Glucose concentrations were determined using the Glucose (GO) Assay kit (Sigma) according the manufacturer’s instructions.

**Short-chain FA analysis by GC and GC-MS.** Short-chain FAs from anaerobic cell culture supernatants were detected and quantified using a Varian 450 gas chromatograph fitted with an Agilent HP-5MS column and flame-ionization detector. Supernatants were filtered using a Nalgene 0.2 μm syringe filter and 1 μl was injected into the chromatograph. The method used a 10:1 split ratio and a 30 ml min−1 flow rate with helium as the carrier gas. The oven was held at 40 °C for 1 min followed by a ramp of 10 °C min−1 up to 240 °C at which point the temperature was held at 240 °C for 15 min.

For analysis by GC-MS, 0.5 ml of culture supernatant was acidified with 50 μl 1 M HCl and extracted with 1 ml of chloroform/methanol (1:1) (Mayer & Shanklin, 2007) using heptanoic acid as an extraction control where indicated. The organic phase (1 μl) was injected onto an Agilent 6890 Series gas chromatograph equipped with an Agilent HP-5MS column and an Agilent single quadrupole 5973 mass spectrometer using electron ionization. The method used a splitless injection with a 50 ml min−1 flow rate and helium as the carrier gas, and a mean velocity of 36 cm s−1. Oven temperature and run times were identical to GC flame-ionization detector analysis described above.

**RESULTS**

**Enzymes for the fermentative production of even-chain FAs by E. coli**

The enzymes used for fermentative production of even-chain FAs by E. coli were thiolase from C. kluyveri (CkThl), 3-hydroxybutyryl-CoA dehydrogenase from C. beijerinckii (CbHbd), coenzyme from C. kluyveri (CkCrt) and trans-enoyl-CoA reductase from E. gracilis (EgTer) (see Methods). These enzymes are expected to catalyse production of FAs by the pathway shown in Fig. 1. The clostridial enzymes used were chosen from organisms that naturally produce butyrate and hexanoate by fermentation with the intention of producing longer-chain FAs (Seedorf et al., 2008). The C. beijerinckii Hbd was chosen because it is NAD-specific (Colby & Chen, 1992) and E. gracilis Ter was used because prior studies indicated that this enzyme improved production of butyrate and 1-butanol and that it is capable of reducing longer-chain enoyl-CoA compounds (Bond-Watts et al., 2011; Shen et al., 2011). All genes were obtained by gene synthesis and codon-optimized for protein production in E. coli.

**Specific activities of enzymes for even-chain FA production in E. coli**

To determine whether the enzymes chosen for even-chain FA production could be produced from codon-optimized genes with high activity in E. coli, we overproduced and purified his-tagged versions (Fig. 2). The specific activities
pathways deleted to reduce the formation of undesired by-products (Jarboe et al., 2007). The activities of the CkThl, CbHbd, CkCrt and EgTer' enzymes within the context of the synthetic operon were then measured in crude cell extracts, and found to be 10.4, 39.3, 1259.2 and 1.6 μmol min⁻¹ mg⁻¹, respectively. These activities are sufficiently high to allow the production of substantial quantities of FAs. Thus, the resulting strain (BE1576) was expected to produce even-chain FAs by the pathway shown in Fig. 1 with reduced by-product formation due to genetic deletion of competing native fermentation pathways.

### FA production via fermentation

To test E. coli strain BE1576 for FA production by fermentation, cells were grown aerobically and then resuspended at an OD₆₀₀ of about 20 under anaerobic conditions. Under these conditions BE1576 did not grow appreciably and was functioning as a cell catalyst. When cells were incubated with 0.4 % glucose in M9 minimal medium for 20 h, HPLC analyses showed that the culture medium contained 0.815 ± 0.005 g butyrate l⁻¹ and 0.395 ± 0.002 g hexanoate l⁻¹. In addition, GC analyses were able to detect 0.051 ± 0.007 g octanoate l⁻¹. During these fermentations, about 93 % of the glucose was utilized. Control strains having plasmid without insert did not produce detectable amounts of FAs. Thus, the results indicated that strain BE1576 produced significant quantities of butyrate, hexanoate and octanoate by fermentation of glucose.

### GC-MS to verify the FAs produced

Initial identification of butyrate, hexanoate and octanoate in culture media was based on HPLC retention times. To verify the identity of these fermentation products, GC-MS was performed on FAs extracted from culture medium using chloroform/methanol (Mayer & Shanklin, 2007). The total ion chromatogram showed peaks at 10.3, 12.6 and 15.0 min, which matched the retention times of the butyrate, hexanoate and octanoate standards (Fig. 3). In addition, the mass spectrum from each peak was used to query the National Institute of Standards and Technology library and results showed near-perfect matches to spectra for butyrate, hexanoate and octanoate (Fig. 3). No FA peaks were detected in assays containing control cells lacking the synthetic operon for even-chain FA production. These results confirmed that butyrate, hexanoate and octanoate accumulated in the culture medium.

### Effect of excess glucose

In the experiments described above (which used 0.4 % glucose) >90 % of the glucose was consumed. To test whether additional glucose would increase the production of C₄, C₆ and C₈ FAs, fermentations were done using 2 % glucose in both M9 and MB media. Cells incubated in M9 media for 24 h accumulated 1.632 ± 0.008 g butyrate l⁻¹, 0.586 ± 0.011 g hexanoate l⁻¹ and 0.028 ± 0.001 g

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**Fig. 2.** Purification of the enzymes used for the production of even-chain FAs. SDS-PAGE was used to assess protein purity. The gel was stained with Coomassie. Three micrograms of purified protein of the N-terminally 6 × his-tagged enzymes were 165.2, 438.5, 5224.7 and 76.5 μmol min⁻¹ mg⁻¹ for CkThl, CbHbd, CkCrt and EgTer', respectively. The specific activities for CkThl and CbHbd are somewhat higher than previously reported activities of 115 and 41.6 μmol min⁻¹ mg⁻¹, respectively (Sliwkowski & Hartmanis, 1984; von Hugo et al., 1972). The activity of CkCrt is similar to that reported for the C. acetobutylicum enzyme (6155 μmol min⁻¹ mg⁻¹) (Waterson & Hill, 1972). With respect to EgTer, the cited specific activity for the recombinant enzyme in crude extracts was 3.7 μmol min⁻¹ mg⁻¹ (Shen et al., 2011). The specific activity determined in this study for the purified enzyme is approximately 19 times higher (76.5 μmole⁻¹ min⁻¹ mg⁻¹); thus, these activities are likely to be roughly similar for purified enzyme.

**Strain engineering for even-chain FA production**

The optimized genes described above were used to construct a synthetic operon for the production of the CkThl, CbHbd, CkCrt and EgTer' enzymes. This operon was moved into strain ZH84 (E. coli K-12 MG1655 DE3, ΔldhA, ΔadhE, ΔackA, Δpta, ΔfrdC) which has the native fermentation
When incubated in TB medium, the short-chain FA production after 24 h incubation was $1.312 \pm 0.009$ g butyrate l$^{-1}$, $0.686 \pm 0.015$ g hexanoate l$^{-1}$ and $0.216 \pm 0.108$ g octanoate l$^{-1}$. Somewhat higher hexanoate and substantially more octanoate was produced on TB medium compared with on M9. This suggests that the carbon sources present in TB provided additional reducing power (NADH) for the production of hexanoate and octanoate. For each of the experiments described above, control strains having plasmid without insert did not produce detectable amounts of butyrate, hexanoate or octanoate. Somewhat unexpectedly, under the conditions used, only about 25% of the added glucose was consumed.

**Time-course of butyrate production**

We also looked at the time-course of butyrate formation by strain BE1576 using TB medium with 2% glucose. Results showed that butyrate accumulated rapidly for the first hour, reaching a level of $0.79 \pm 0.05$ g l$^{-1}$ then levelled off abruptly increasing to a final yield of $1.77 \pm 0.05$ g l$^{-1}$ after 24 h (Fig. 4). Again, less than 25% of the added glucose was consumed. It seemed possible that the fall off in FA production and the incomplete glucose consumption may have been due to FA toxicity inhibiting the metabolism of the production strain, BE1576 (Nunn et al., 1979). Given that low pH increases the toxicity of FAs, we looked at butyrate production on TB plus 2% glucose while controlling the pH in a range between 7.0 and 7.1. This was done over a 24 h period, followed by anaerobic incubation without pH control for an additional 20 h. After the initial 24 h period, 68.18% of the glucose had been consumed and butyrate, hexanoate and octanoate had been produced at 7.47, 0.37 and 0.043 g l$^{-1}$, respectively. Following the additional 20 h incubation period, 82.7% of the glucose had been consumed and the FA titres were at 9.67, 0.365

**Fig. 3.** Identification of butanoic, hexanoic and octanoic acids by GC-MS. Top: elution times were 10.3 (butanoic), 12.6 (hexanoic) and 15 min (octanoic). Middle row, left to right: spectra for butanoic, hexanoic and octanoic acids produced by fermentation. Bottom row, left to right: spectra for butanoic, hexanoic and octanoic acids obtained from the National Institute of Standards and Technology library.
and 0.0425 g l⁻¹, respectively. The reason that the relative production of butyrate, hexanoate and octanoate varied with and without pH control (see above) is uncertain. The medium pH might affect thioesterase expression but alternative explanations are also possible. More importantly, however, pH control substantially increased FA production.

Involvement of native thioesterases in FA production by strain BE1576

In the studies described above, no recombinant thioesterases were produced in the FA production strain (BE1576). Thus, although other possibilities were not ruled out, it seemed likely that hydrolysis of acyl-CoA compounds to FAs was mediated by endogenous thioesterases. To test for a possible role of native thioesterases in the production of FAs, we made single gene knockout mutations in four genes that are annotated as thioesterases in E. coli: tesB, paal, yciA and ybgC. Each mutation was moved individually into FA production strain BE1576 and its effect on fermentative FA production was tested (Fig. 5). Deletion of the tesB, paal or ybgC genes had little effect on the fermentative production of FA. However, deletion of the yciA gene substantially decreased butyrate formation while increasing hexanoate formation. This suggested that YciA plays a significant role in the hydrolysis of butyryl-CoA to butyrate.

Production of odd-chain FAs

The enzymes used for the production of even-chain FAs described above (CktHl, ChHbd, CkCrt and EgTer’) are also expected to mediate the production of odd-chain FAs, if a source of propionyl-CoA is provided. In this case, propionyl-CoA will condense with acetyl-CoA in the first step of the pathway to produce 3-ketovalerate, which will proceed through the pathway similarly to its C4 analogue (Fig. 1) and produce pentanoate. To produce propionyl-CoA, we chose a coenzyme B₁₂-dependent pathway. 1,2-Propanediol can be converted to propionyl-CoA by the sequential action of coenzyme B₁₂-dependent diol dehydratase (PduCDE) and HS-CoA-dependent propionaldehyde dehydrogenase (PduP) (Fig. 6). This pathway is potentially advantageous because 1,2-propanediol can be efficiently produced from glucose (Clomburg & Gonzalez, 2011). Accordingly, we cloned the genes for diol dehydratase (pduCDE), diol dehydratase reactivase (pduGH) and propionaldehyde dehydrogenase (pduP) from Salmonella enterica into pBE522 as a synthetic operon. To determine whether this operon produced active enzymes in E. coli, we measured the activities of SePduCDE, SePduGH and SePduP in crude cell extracts of strain BE1908. The activities of the PduCDE diol dehydratase and the PduP aldehyde dehydrogenase were 51.0 and 9.2 μmol min⁻¹ mg⁻¹, respectively. There is no quantitative assay for reactivation of PduCDE by PduGH; however, we found that the expression plasmid was able to complement a pduGH deletion mutant of S. enterica for growth on 1,2-propanediol, establishing the active expression of the SePduGH reactivase (data not shown). Given the measured activities of SePduCDE, SePduP and SePduGH, we expected this operon to effectively mediate the production of propionyl-CoA from 1,2-propanediol.

Next, we combined plasmids for the production of even-chain FAs (pBE1570-thl-hbd-ccr-ter) and for propionyl-CoA production (pBE522-pduP-pduCDE-pduGH) into a background where the native fermentation pathways were deleted. The resulting strain (BE1952) was assayed for FA production by fermentation using TB medium supplemented with 2 % glucose, 1 % 1,2-propanediol and 200 nM coenzyme B₁₂. HPLC analyses showed the production of propionate (1.19 ± 0.23 g l⁻¹) butyrate (0.23 ± 0.03 g l⁻¹) and pentanoate and (0.20 ± 0.1 g l⁻¹). In addition, GC analyses found a small amount of heptanoate (0.008 ± 0.004 g l⁻¹). The authenticity of the pentanoate and heptanoate were verified by GC-MS (as described above). Both

![Figure 4](http://mic.sgmjournals.org)  
**Fig. 4.** Time-course of fermentative butyrate production. Butyrate production began rapidly but quickly levelled off, suggesting that productivity was limited by product toxicity or medium acidification. The strain used for butyrate production was BE1576 (Table 1). The medium was TB supplemented with 2 % glucose.

![Figure 5](http://mic.sgmjournals.org)  
**Fig. 5.** Effects of thioesterase deletions on the fermentative production of butyrate (light grey) and hexanoate (dark grey). The strains used were BE1576, BE1869, BE1871, BE1872 and BE1873 (Table 1). The medium was TB supplemented with 2 % glucose.
DISCUSSION

Three general approaches for the microbial production of FAs have been studied most extensively: early termination of lipid biosynthesis, reverse β-oxidation and fermentation (Clomburg et al., 2012; Dellomonaco et al., 2010, 2011; Lennen & Pfleger, 2012; Peralta-Yahya et al., 2012; Steen et al., 2010; Zhang et al., 2009). Prior studies of the fermentative production of FAs used clostridia to produce high levels of butyrate or hexanoate from glucose or galactitol, respectively (Jiang et al., 2010; Wei et al., 2013). However, clostridia are disadvantageous due to slow growth, a requirement for strictly anaerobic culture conditions, sporulation and relatively limited genetic tools for use in strain improvement and product diversification (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). To circumvent these problems, E. coli was engineered for fermentative butyrate production (Baek et al., 2013; Lim et al., 2013; Seregina et al., 2010). This was done by deleting E. coli’s native fermentation pathways and engineering it to produce enzymes for butyrate formation that catalyse the condensation and reduction of acyl-CoA compounds: AtoB and TesB from E. coli, Hbd and Crt from C. acetobutylicum and Ter from Treponema denticola (Baek et al., 2013; Fischer et al., 2010; Lim et al., 2013; Zhang et al., 2009). Here, we used an E. coli strain deleted for the native fermentation pathways (ΔadhE::FRT, ΔldhA::FRT, ΔackA-pta::FRT, ΔfrdC::FRT) and an analogous set of enzymes for fermentative FA production by E. coli (CKThl-CHHbd-CKCrt-Eg(Ter)) (strain BE1576). However, in the studies reported here, not only was butyrate produced but also hexanoate and octanoate. This was probably due to the substrate specificities of the particular enzymes used as prior studies aimed at the production of fuel alcohols by an analogous pathway found that the enzymes used for acyl-CoA condensation and reduction affected the amounts of longer-chain alcohols produced (Dekishima et al., 2011). The yields of butyrate reported here are similar to the levels previously obtained in E. coli, 9.670 versus 7.2 g l⁻¹ in prior studies (Baek et al., 2013). The highest levels of hexanoate produced here were about 10-fold higher than prior studies, 1.963 versus about 0.180 g l⁻¹ (Machado et al., 2012). The highest amount of octanoate measured here was 0.216 g l⁻¹ but in prior studies octanoate levels were not reported. Thus, the system reported here provides a reasonable basis for the production of even-chain FAs in E. coli including some longer-chain molecules.

In this report, we produced odd-chain FAs using a new pathway that has some potential advantages compared with prior methods (Fig. 6). The production of odd-chain FAs starts with the condensation of propionyl-CoA and acetyl-CoA by a coenzyme B₁₂-dependent reverse diol dehydratase (PduCDE) and propionaldehyde dehydrogenase (PduP). The propionyl-CoA formed is condensed with acetyl-CoA to from 3-keto-pentanoate, which is converted to pentanoic acid by the enzymes shown in Fig. 1.
potentially advantageous due to fewer steps and a lower energy cost. The amounts of pentanoate and heptanoate produced here by combining enzymes for acyl-CoA condensation with the B12-dependent pathway of propionyl-CoA formation were 0.201 ± 0.01 and 0.008 g l⁻¹, respectively. In prior studies that produced propionyl-CoA via threonine, the combined amounts of C5 compounds (pentanoate and pentanol) produced from glucose or glycerol were about 0.03 or 0.2 g l⁻¹, respectively. To our knowledge, heptanoate has not been previously produced in E. coli by fermentation. Thus, the approach reported here appears to be a promising basis for further development of odd-chain FA production not only by fermentation, but also by premature termination of lipid biosynthesis or reverse β-oxidation.

A notable difference between the system for FA production described here and prior systems was that no thioesterase activity was engineered into the strains described here. Thus, the cleavage of acyl-CoA derivatives to the corresponding FAs was catalysed by endogenous enzyme systems. Activity was engineered into the strains described here and prior systems was that no thioesterase was eliminated by the use of resistant production strains or fermentation conditions. The amounts of pentanoate and heptanoate produced from glucose or glycerol were about 0.03 or 0.2 g l⁻¹, respectively. To our knowledge, heptanoate has not been previously produced in E. coli by fermentation. Thus, the approach reported here appears to be a promising basis for further development of odd-chain FA production not only by fermentation, but also by premature termination of lipid biosynthesis or reverse β-oxidation.

In the studies described here, we also looked at the time-course of even-chain FA production. Results showed a relatively high initial rate of butyrate production for the first hour: 0.79 g l⁻¹ h⁻¹ (0.2 g l⁻¹ h⁻¹ per gram of dry cells), which is about 10% of a commercially meaningful rate. The rapid decline in the rate of FA production a later time points was probably due to the accumulation of FAs that are known to be toxic or to medium acidification (Desbois & Smith, 2010; Lennen & Pfleger, 2012; Royce et al., 2013). We also found that pH control (FAs are more toxic at a lower pH) substantially increased total FA production (butyrate + hexanoate + octanoate) by about 450%. Thus, it is likely that the fermentative production of FAs by E. coli is mainly limited by product toxicity and/or medium acidification and that the system used here could support substantially higher levels of FA production if this difficulty could be reduced or eliminated by the use of resistant production strains or fermentation processes that continuously remove product.

ACKNOWLEDGEMENTS

This paper is based upon work supported by the National Science Foundation Award No. ECC-0813570. We thank the ISU DNA Sequencing and Synthesis facility for assistance with DNA analyses. We thank Ann Perera and the W. M. Keck Metabolomics Research Laboratory for assistance with GC-MS.

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