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Engineering *Escherichia coli* for odd straight medium chain free fatty acid production

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Abstract Microbial biosynthesis of free fatty acids (FFAs) can be achieved by introducing an acyl–acyl carrier protein thioesterase gene into *Escherichia coli*. The engineered *E. coli* usually produced even chain FFAs. In this study, propionyl-CoA synthetase (*prpE*) from *Salmonella enterica* was overexpressed in two efficient even chain FFAs producers, ML103 (pXZM12) carrying the acyl-ACP thioesterase gene from *Umbellularia californica* and ML103 (pXZ18) carrying the acyl-ACP thioesterase gene from *Ricinus communis* combined with supplement of extracellular propionate. With these metabolically engineered *E. coli*, the odd straight chain FFAs, undecanoic acid (C11:0), tridecanoic acid (C13:0), and pentadecanoic acid (C15:0) were produced from glucose and propionate. The highest total odd straight chain FFAs produced by ML103 (pXZM12, pBAD-*prpE*) reached 276 mg/l with a ratio of 23.43 % of the total FFAs. In ML103 (pXZ18, pBAD-*prpE*), the highest total odd straight chain FFAs accumulated to 297 mg/l, and the ratio reached 17.68 % of the total FFAs. Due to the different substrate specificity of the acyl-ACP thioesterases, the major odd straight chain FFA components of ML103 (pXZM12, pBAD-*prpE*) were undecanoic acid and tridecanoic acid, while the ML103 (pXZ18, pBAD-*prpE*) preferred pentadecanoic acid.

Keywords Fatty acid production · Odd straight chain · *prpE* · *propionate* · *Escherichia coli*

Introduction

Microbial biosynthesis of green sustainable biofuels or biochemicals from renewable feedstocks has attracted significant attention in recent years. Free fatty acids (FFAs) can be used as precursors for the production of biofuels or biochemicals (Nikolau et al. 2008; Lennen et al. 2010; Liu et al. 2010; Steen et al. 2010; Handke et al. 2011). The odd straight-chain FFAs and derivatives are used in the manufacture of agricultural chemicals, flavor and fragrance intermediates, pharmaceuticals, cosmetics, plasticizers, coatings, and industrial chemicals (Telos et al. 1999; Clausen et al. 2010; Köckritz et al. 2010). Furthermore, biodiesel is considered as an alternative to diesel fuel, which is produced from petrochemical feedstocks and can be synthesis by FFAs esterification (Nawabi et al. 2011) and triglycerides (TG) transesterification with short chain alcohols (Li et al. 2012a, b). Different FFA compositions, which can be obtained by adding different quality and quantity of odd straight-chain FFAs to regular FFAs, will be useful for further modification of the biodiesel quality (Knothe 2005, 2008, 2009).

FFAs are naturally accumulated with a significant quantity in most oleaginous microorganisms or plants (Meng et al. 2009; Wang et al. 2011). However, *E. coli* normally produce FFAs mainly for the biosynthesis of lipids and cell membranes, and it does not accumulate FFAs (Voelker and Davies 1994). The chain numbers of the most of straight FFA produced by microorganisms or plants are even. Only a small amount of odd straight chain fatty acids are produced by certain organisms, such as some stomach, ruminant, and soil bacteria (Ringelberg et al. 1989; Kim et al. 2005; Or-Rashid et al. 2007).

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The fatty acid biosynthesis pathways together with a simplified central aerobic metabolic pathway of *E. coli* using glucose as a carbon source are shown in Fig. 1. The first committed step of fatty acid elongation cycle is the conversion of acetyl-CoA and malonyl-ACP to acetoacetyl-ACP through the enzyme β-ketoacyl-ACP synthase III (KAS III, *fabH*). FFAs can be produced by introducing an acyl–acyl carrier protein (ACP) thioesterase (TE) gene into *E. coli*. The presence of the acyl-ACP TE breaks the fatty acid elongation cycle and releases FFAs (Lu et al. 2008; Li et al. 2012a; Zhang et al. 2011; Youngquist et al. 2013). Ingram and colleagues (1977) used propionate to induce synthesis of odd-chain-length fatty acids by wild type *Escherichia coli* K-12. Their research indicated that KAS III might possess the ability to utilize propionyl-CoA as a substrate instead of acetyl-CoA. Tseng and Prather (2012) engineered and optimized three modular metabolic pathways, which was validated separately and then assembled together, for the synthesis of odd short chain molecules such as propionate, *trans*-2-pentenoate, valerate, and pentanol. However, the longest chain length of these molecules is five, much shorter than those products of the native FFAs synthesis cycle. The *E. coli* strains with engineered reversal of the β-oxidation cycle can also produce C7, C9 and C11 fatty alcohols when propionate was added in the medium (Dellomonaco et al. 2011).

In this study, we investigated the biosynthesis of odd straight medium chain FFAs with various chain lengths based on the native FFAs synthesis cycle in two efficient FFA production strains, which were engineered in our laboratory by overexpression of acyl-ACP TE genes from *Ricinus communis* and *Umbellularia californica*, to produce, respectively. Furthermore, it has been shown in previous studies that the expression of propionyl-CoA synthetase (*prpE*) from *Salmonella enterica* can improve intracellular propionyl-CoA availability, and this finding was successfully applied to PHBV production (Aldor and Keasling 2001; Wong et al. 2008). We examined the effect of the overexpression of propionyl-CoA synthetase on odd straight chain FFAs production. The result showed that the amount of odd straight chain FFAs accumulated depends on the combination of propionate supplementation and propionyl-CoA synthetase employed. In addition, the distribution of FFAs is correlated with the acyl-ACP TE used.

Materials and methods

Strains and plasmids

The *E. coli* strain ML103 (Zhang et al. 2011; Li et al. 2012b), a MG1655 derivative, was used as the host strain in this study.

Plasmid construction

For the construction of pBAD-*prpE*, the plasmid pMMB-*prpE* (Aldor and Keasling 2001; Wong et al. 2008), containing *S. enterica prpE* gene, was used as templates for amplification of *prpE*. The *prpE* gene was amplified by polymerase chain reaction (PCR) from pMMB-*prpE* using the forward primer 5′-GCGGGTACCAGGAGGTTTTTATTATGTCTTTTAGCG-3′ and the reverse primer 5′-GCGCAAGCTTCTATTCTT

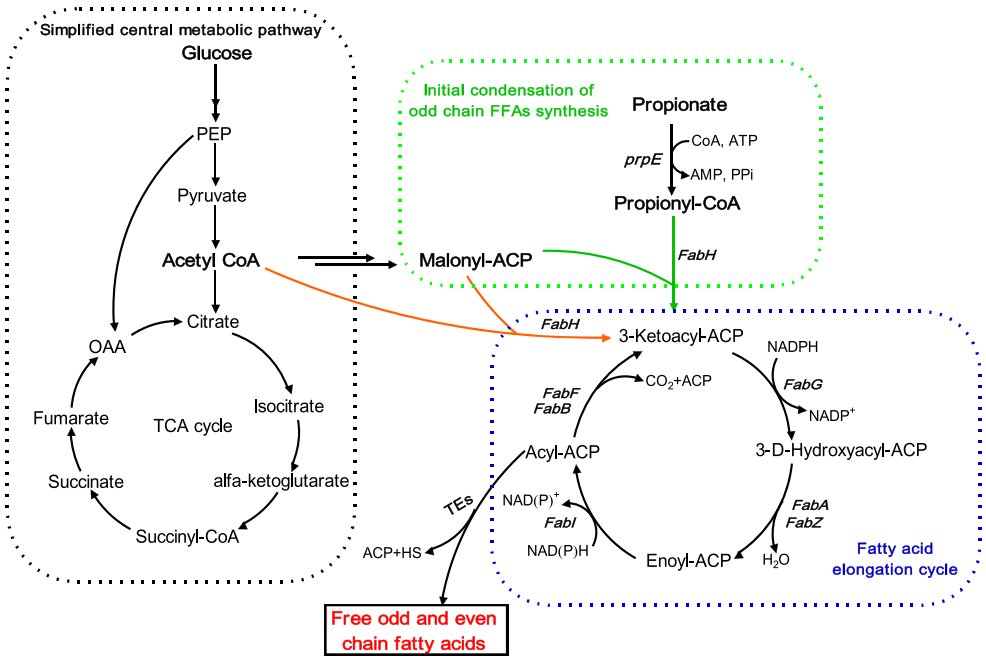

Fig. 1 Metabolic pathways of *Escherichia coli*, including simplified central aerobic pathway and the FFAs production pathway. The simplified central aerobic pathway is shown in *black dashed box*. The substrate of initial condensation of odd chain FFAs synthesis was changed to propionyl-CoA rather than acetyl-CoA and is shown in the *green dashed box*. The *orange lines with arrow* show the initial condensation of even chain FFAs synthesis. The elongation of fatty acid synthesis cycle is shown in the *blue dashed box*

CGATCGCCTGGCGAATTTG-3′. The resulting PCR product was subsequently cloned into plasmid pBAD33 as a 1.9-kb *Kpn*I–*Hin*dIII (underlined) fragment to form pBAD-*prpE*. The plasmid pXZ18, carrying an acyl-ACP TE gene from *R. communis*, and pXZM12, carrying an acyl-ACP TE gene from *U. californica* were constructed by the previous member of our laboratory (Zhang et al. 2011; San et al. 2011). A list of the strain and plasmids in this study is shown in Table 1.

Culture medium and conditions

During strains construction, cultures were grown at 37 °C in Lysogeny Broth (LB) (per liter: tryptone 10 g, yeast extract 5 g, sodium chloride 10 g) (Bertani 1951), and appropriate antibiotics were included at the following concentrations: ampicillin, 100 μg/ml; chloramphenicol, 34 μg/ml.

The primary preculture was prepared by inoculating a single colony from a freshly grown plate in 5 ml of LB medium in an orbital shaker (New Brunswick Scientific, New Jersey, USA) operated overnight at 250 rpm and 37 °C. The secondary preculture was prepared by inoculating 250-ml flasks containing 50 ml LB medium with 0.5 ml primary preculture and culturing at 37 °C and 250 rpm for 9 h. The cells were harvested aseptically by centrifugation at 3,300×*g* for 6 min, and resuspended in the appropriate volume of fresh fermentation medium which was calculated based on the inoculation size of 10 %. LB broth medium supplemented with 15 g/l of glucose were used for aerobic cultivations. Two concentrations of propionate, 8 and 12 mM, were added to the fermentation medium initially, and the initial pH was adjusted to 7.5. All of the media were supplemented with 100 mg/l ampicillin and 34 μg/ml chloramphenicol. The expression of the acyl-ACP TEs of *R. communis* (pXZ18) and *U. californica* (pXZM12) were induced by the addition of isopropyl-β-D-thiogalactopyranoside (IPTG) to the final concentrations of 500 and 200 μM, respectively. The expression of the propionyl-CoA synthetase from *S. enterica* was induced by different concentrations of arabinose. The cells were then cultivated in an orbital shake (New Brunswick Scientific) operated at 250 rpm and 30 °C. Samples were taken at 24 and 48 h for fatty acids and metabolic analysis. All experiments were carried out in triplicate.

Analytical methods

Cell density was estimated by measuring the optical density of appropriately diluted culture samples at 600 nm (Bausch & Lomb Spectronic 1001); the culture was diluted to the linear range with 0.95 % (w/v) NaCl. Previously established HPLC methodology was used to analyze glucose and the extracellular metabolites (Dittrich et al. 2005; Lin et al. 2005). Briefly, 1 ml of culture was centrifuged and the supernatant was then filtered through a 0.22-μm syringe filter for HPLC analysis. The HPLC system (Shimadzu-10A Systems; Shimadzu, Colombia, MD, USA) used was equipped with a cation-exchange column (HPX-87H, Bio-Rad Labs, California, USA), a UV detector (Shimadzu SPD-10A) and a differential refractive index (RI) detector (Waters 2410; Waters, Milford, MA, USA). A mobile phase of 2.5 mM H_2SO_4 solution at a 0.5 ml/min flow rate was used, and the column was operated at 55 °C. Metabolites such as glucose, acetate, ethanol and lactate were measured by the RI detector and pyruvate was measured by the UV detector at 210 nm.

Fatty acid analysis

Cell cultures were harvested and prepared for fatty acid analysis, as described earlier (Voelker and Davies. 1994; Li et al. 2012b). Authentic standards were used to quantify individual fatty acids; heptadecanoic acid (C17:0) was added as an internal standard in all samples. A brief description of fatty acid extraction is provided below. Then, 1 ml of cell broth was taken into a 16×150 mm kimax glass tube. Next, 100 μl of pure acetic acid and 200 μl of heptadecanoic acid solution (1 g/l of heptadecanoic acid in $CHCl_3$) were added into the same tube sequentially. The mixture was mixed by vortexing. Then 4 ml of $CHCl_3/CH_3OH$ mixture (1:2, v/v) was added into the tube. The tube with cap was put on a vertexer to mix

Table 1 List of strains and plasmids used in this study

Strain plasmids and primers	Relevant genotype or description	Source or reference
Strain		
ML103	*F⁻ lambda⁻ ilvG⁻ rfb⁻ rph⁻ fadD⁻*	Zhang et al. 2011; Li et al. 2012b
Plasmids		
pBAD33	Cloning vector, Cm resistant, pACYC18 origin vector	Guzman et al. 1995
pBAD-*prpE*	pBAD33 carries an a propionyl-CoA synthetase from *Salmonella enterica*	This study
pXZ18	pTrc99a carries an acyl-ACP thioesterase from *Ricinus communis*	Zhang et al. 2011
pXZM12	pTrc99a carries an acyl-ACP thioesterase from *Umbellularia californica*	San et al. 2011

well before placing into an incubator at 60–65 °C for 30 min. After the heating step, 2.5 ml of pure $CHCl_3$ and 2.5 ml of Super-Q water (Millipore, Massachusetts, USA) was added to the tube sequentially. The tube was put on a vertexer to mix well, and then centrifuged for 5 min at 5,000 rpm. The mixture in the tube would separate into two layers. The lower layer, $CHCl_3$ layer, was recovered by pass through 1 g anhydrous sodium sulfate in 5.75-inch glass Pasteur pipet, and collect in a 13×100 mm test tube for GC-MS analysis.

The fatty acid content of each sample was quantified by GC-FID/MS system (GC/MS QP 2010 from Shimadzu Scientific) using a single quadrupole mass spectrometer (MS) with an electron impact ionization (EI) source and a flame ionization detector (FID). The GC-FID/MS system was also equipment with two auto-injectors; two identical DB-5MS columns (30 m×0.25 mm×0.25 μm; Agilent Co., USA) were directly connected to either the FID or the MS. During the analysis, two identical samples were injected to each column individually and sequentially. We found out that this configuration yielded better quality data than the single column split detector configuration. Helium was used as the carrier gas and the flow rate was set at 1 ml/min. The oven temperature was initially held at 50 °C for 2 min. Thereafter, the temperature was raised with a gradient of 4 °C/min until the temperature reached 220 °C. This temperature was then held for 10 min. Other system settings were as follows: 280 °C interface temperature, 250 °C ion source temperature, and electron EI was set 0 kV relative to the tuning result. Mass spectra were analyzed by full scan mode. Raw MS data were processed using the program GC-MS post-run analysis to obtain a spectrum and to identify a peak together with running the standards.

Results

Effect of propionate on odd straight chain fatty acid production

The strategy of using propionyl-CoA instead of acetyl-CoA as a substrate of the first step in fatty acid elongation cycle was examined to produce odd straight chain fatty acid in efficient FFA producers, ML103 (pXZM12) and ML103 (pXZ18) (Zhang et al. 2011). Since the native *prpE* of *E. coli* has the ability to convert propionate to propionyl-CoA (Ingram et al. 1977; Brock et al. 2002), 8 and 12 mM of propionate were added to the fermentation medium to investigate the effect of propionate on the odd straight chain fatty acid production. The strain ML103 (pXZM12) and ML103 (pXZ18) with the background plasmid pBAD33 were used as the control (Figs. 2 and 3, Table 2).

Without propionate in the medium, no odd straight chain FFAs were produced in the control strains ML103 (pXZM12, pBAD33) and ML103 (pXZ18, pBAD33) (Fig. 3a). It was similar to the result using wild type *E. coli* K-12 strain (Ingram et al. 1977). With the addition of propionate, the strains ML103 (pXZM12, pBAD33) and ML103 (pXZ18, pBAD33) accumulated very low levels of odd straight chain FFAs while accumulating even chain FFAs as major products (Fig. 3, Table 2). In the case of ML103 (pXZM12, pBAD33) with 8 mM propionate, undecanoic acid and tridecanoic acid were produced as the major components of odd straight chain fatty acid (Fig. 2). The concentrations of undecanoic acid, tridecanoic acid, and pentadecanoic acid accumulated to 10.33, 19.36 and 1.83 mg/l at 24 h (Fig. S1a), respectively. At 48 h, the concentrations of undecanoic acid and tridecanoic acid increased by 66.7 % and 44.8 % while the concentration of pentadecanoic acid dropped by 8.98 % (Fig. 2a). The percentage of total odd straight chain fatty acids reached around 3.21 % and changed a little at 24 and 48 h (Fig. 3c, Fig. S2c). With a higher concentration of propionate (12 mM) supply, the percentage of total odd straight chain fatty acids increased to 15.43 % at 24 h, about 4.8 times that of 8 mM propionate, and dropped to 12.62 % at 48 h due to the further increase of total even chain fatty acid (Fig. 3c, Fig. S2c). The final concentrations of odd straight chain FFAs reached 178 mg/l (Fig. 3a). For the strain ML103 (pXZ18, pBAD33), pentadecanoic acid was produced with concentrations of 29.22 and 45.1 mg/l at 24 and 48 h under the condition of 8 mM propionate supplementation, respectively (Fig. 2b, Fig. S1b). In the condition of 12 mM propionate addition, tridecanoic acid and pentadecanoic acid became the major odd straight chain FFAs. However, the highest percentage of total odd straight chain fatty acids reached around 3.94 %, only about 25.5 % of the highest value of the strain ML103 (pXZM12, pBAD33) (Fig. 3c). The total FFAs at 48 h reached 2,010 and 1,990 mg/l in 8 and 12 mM propionate conditions, respectively, which were 1.48 (1,360 mg/l) and 1.41 (1411 mg/l) times of ML103 (pXZM12, pBAD33) (Fig. 3b). ML103 (pXZ18, pBAD33) seemed to prefer even chain FFA production more than that of ML103 (pXZM12, pBAD33) when propionate was added to the fermentation media.

Compared to the conditions with and without propionate in the media, the glucose utilization at 24 h were similar for both ML103 (pXZM12, pBAD33) and ML103 (pXZ18, pBAD33) strains; all glucose was consumed within 48 h (Fig. S3). Different acetate accumulations were observed for both strains. Under the condition with no propionate, a large amount of acetate was accumulated; ML103 (pXZM12, pBAD33) accumulated about 8.15 and 6.58 mM at 24 and 48 h, and ML103 (pXZ18, pBAD33) accumulated about and 24.5 and 38.2 mM at 24 and 48 h. While adding propionate in both strains reduced the acetate accumulation (Fig. S3). One of the possible reasons was that appropriate propionate may have a positive effect on reducing the overflow metabolism, and that the acetyl-CoA production rate matched well with

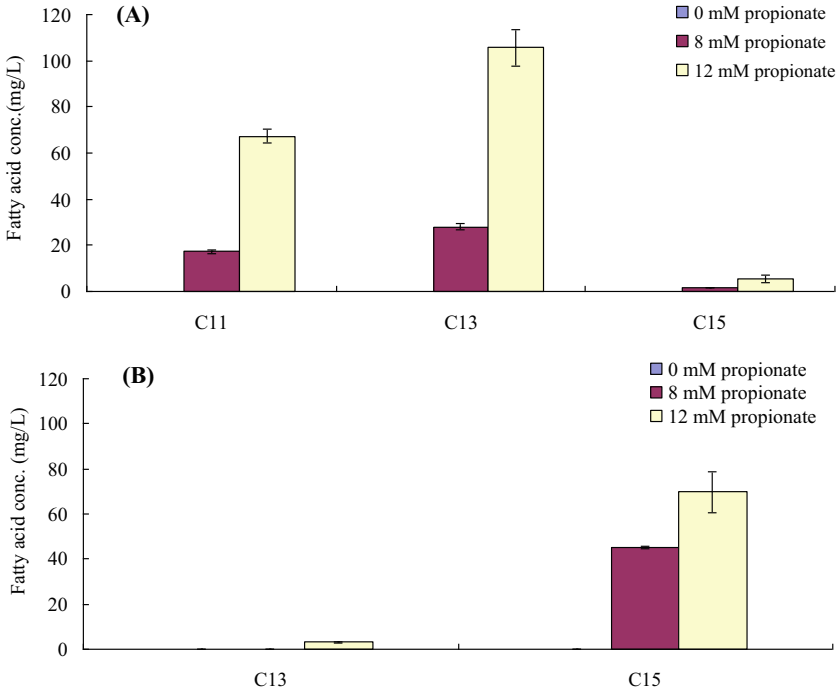

Fig. 2 Effect of different initial propionate concentration on odd straight chain FFAs distribution in ML103 (pXZM12, pBAD33) and ML103 (pXZ18, pBAD33) at 48 h. **a** ML103 (pXZM12, pBAD33); **b** ML103 (pXZ18, pBAD33)

that of fatty acid elongation through the formation of malonyl-CoA. Without propionate in the media, the optical density of ML103 (pXZM12, pBAD33) changed a little with time, however the optical density of ML103 (pXZ18, pBAD33) increased from 10.19 (24 h) to 13.05 (48 h). When 8 and 12 mM propionate were added, the optical densities of ML103 (pXZM12, pBAD33) were similar at both concentrations at 24 h, and they dropped by 21.3 % and 41.5 % at 48 h, respectively, due to cell autolysis. The optical density of ML103 (pXZ18, pBAD33) was dropped by 38.3 % and 42 % at 48 h.

Effect of *prpE* expression on odd straight chain fatty acid accumulations

The plasmid pMM-*prpE*, carrying the propionyl-CoA synthetase, was a high copy number plasmid, and it was induced by IPTG, which was the same inducer of the TEs. The concentration of IPTG for propionyl-CoA synthetase induction was reported to be at around 25 μM (Wong et al. 2008), which was much lower than that required by full induction of the TEs (>200 μM; Zhang et al. 2011). IPTG, at either 200 or 500 μM, was toxic to the growth of the strains ML103 (pXZM12, pMM-*prpE*) and ML103 (pXZ18, pMM-*prpE*) (data not shown). Due to this toxic effect, the propionyl-CoA synthetase gene, *prpE*, was sub-cloned to a low copy number plasmid, pBAD33, and its expression was under the control of the arabinose promoter. The resulting construct is named pBAD-*prpE*.

In this study, the effect of different concentrations of arabinose (5, 10, 20 and 50 mM) combined with propionate (8 and 12 mM) on the odd straight chain FFAs production were investigated using the strains of ML103 (pXZ M12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*). The FFA distribution and composition under different conditions at 48 h are shown in Fig. 4 and Table 2. The total FFAs, odd straight chain FFAs at 48 h, as well as the percentages of odd straight chain FFAs are shown in Fig. 5. The data of 24 h are summarized in Figs. S4 and S5.

With 8 mM propionate supplementation, the total concentrations of odd straight chain FFAs in ML103 (pXZM12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*) were increased along with increasing doses of arabinose (Fig. 5a). When 20 mM arabinose was added, the total odd straight chain FFA concentrations reached 192 and 183 mg/l at 48 h, respectively. The highest concentration of total odd straight chain FFAs of both strains occurred at 12 mM propionate (Fig. 5a). In ML103 (pXZM12, pBAD-*prpE*), 10 mM arabinose appeared to be the best level leading to a favorable induction of *prpE*; the total odd straight chain fatty acids reached 276 mg/l with a ratio of 23.4 %. In ML103 (pXZ18, pBAD-*prpE*), the condition of 20 mM arabinose showed the best result; 297 mg/l odd straight chain fatty acid was produced with the ratio of 17.7 % at 48 h. Further increasing the concentration of arabinose resulted in a drop in the total concentration and ratio for both strains.

Different doses of arabinose also showed different glucose utilization and acetate accumulation. At 8 mM propionate,

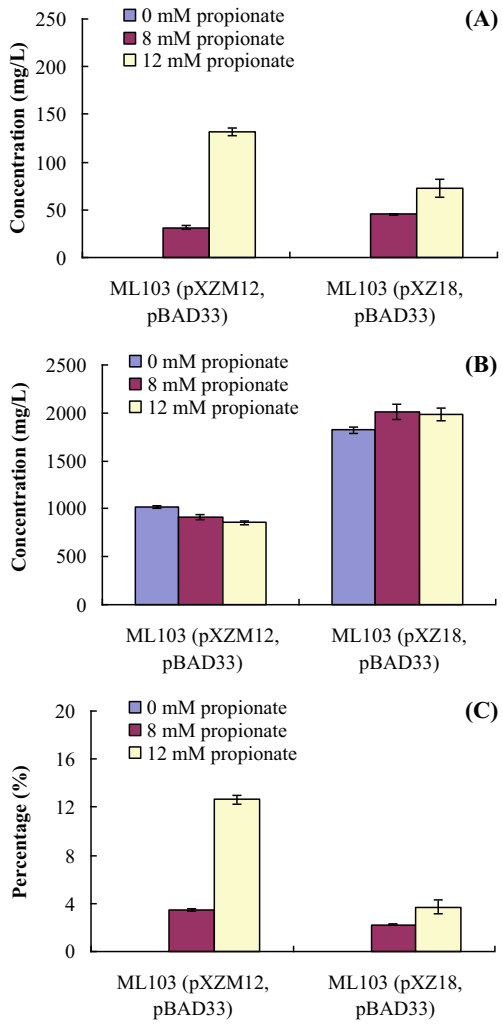

Fig. 3 Effect of different initial propionate concentration on total odd straight chain FFAs and total FFAs produced by ML103 (pXZM12, pBAD33) and ML103 (pXZ18, pBAD33) at 48 h. **a** The total odd chain FFAs; **b** the total FFAs; **c** the percentage of total odd FFAs

glucose consumption by ML103 (pXZ18, pBAD-*prpE*) seemed to be independent of the increased expression of *prpE*, although the consumption dropped slightly in ML103 (pXZM12, pBAD-*prpE*). On the other hand, under the condition of 12 mM propionate, when more arabinoase added, less amount of glucose was consumed by both strains. At the induction level of 50 mM arabinose, only 64.3 61.8 mM glucose were consumed by the strains ML103 (pXZM12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*), respectively at 48 h (Fig. S6a and d). This result also demonstrated that too much expression of *prpE* had a toxic effect on the metabolism of *E. coli*, which is consistent with Wong et al.'s (2008) result. The amount of acetate accumulated by ML103 (pXZM12, pBAD-*prpE*) increased with time and dosage of arabinose reaching the highest level of around 24 mM acetate (Fig. S6b). A large amount of acetate was produced by the strain ML103 (pXZ18, pBAD-*prpE*) at 24 and 48 h (Fig. S6e). The acetate accumulation was unaffected by the different arabinose concentrations except at the 48-h sampling point of 50 mM arabinose with 12 mM propionate, which had about 40 % higher acetate accumulation than that of 10 and 20 mM arabinose (Fig. S6e).

Effect of different thioesterases on FFAs compositions including even and odd straight chain

Two acyl-ACP TEs from *U. californica* and *R. communis*, with different activities on terminating fatty acyl group extension by hydrolyzing the acyl moiety from the acyl-ACP at the appropriate chain length, were examined in this study. The FFA compositions (in percentage of the total FFAs) of ML103 (pXZM12) and ML103 (pXZ18) with and without heterologous *prpE* gene expression at 24- and 48-h sampling times are shown in Table 2.

The FFAs for the control strain ML103 (pXZM12, pBAD33) were mostly composed of dodecenoic acid (C12:1) (10.98 %), dodecanoic acid (C12:0) (71.64 %), tetradecenoic acid (C14:1) (7.57 %), and tetradecanoic acid (C14:0) (3.74 %) straight chain FFAs at 24 h, while the FFAs produced by the strain ML103 (pXZ18, pBAD33) were mainly straight chain tetradecanoic acid (C14:0) (26.47 %), hexadecenoic acid (C16:1) (23.39 %), hexadecanoic acid (C16:0) (36.37 %), and octadecenoic acid (C18:1) (13.87 %). The pattern of both strains did not change much at 48 h. These results were consistent with the previous observations that the acyl-ACP TE from *U. californica* was more efficient for medium chain acyl-ACP (C-12) and that TE from *R. communis* preferred longer chain lengths, C-14 and C-16 (San et al. 2011).

With the addition of propionate (12 mM), the major product of ML103 (pXZM12, pBAD33), dodecanoic acid, dropped significantly, from 71.98 % to 54.7 %, with a corresponding increase in the odd straight chain FFAs at 48 h. The unsaturated dodecenoic acid, however, increased from 10.54 % to 14.63 % while the percentages of tetradecenoic acid and tetradecanoic acid changed a little. The odd straight chain fatty acids, undecanoic acid and tridecanoic acid, increased from non-detectable levels to 4.76 % and 7.48 %, respectively. On the other hand, ML103 (pXZ18, pBAD33) was not affected by the addition of propionate; the ratio of odd straight chain FFAs, tridecanoic acid and pentadecanoic acid were only 0.16 % and 3.5 % of the total FFAs, while the levels of major even chain FFAs remained relatively constant.

Further improvement of odd straight chain FFA production by the introduction of *prpE* exhibited similar effects on the FFA composition of the strains ML103 (pXZ M12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*) compared to the effect of propionate on the control strains. For the strain ML103 (pXZ M12, pBAD-*prpE*), both even chain FFAs,

Table 2 Compositions of free fatty acids of four engineered strains in different conditions

Strain	Time	Condition		Compositions of free fatty acids (%)										
		Propionate (mM)	Arabinose (mM)	C10	C11	C12:1	C12	C13	C14:1	C14	C15	C16:1	C16	C18
ML103 (pXZM12, pBAD33)	24 h	0	0	0.99±0.01	ND	10.98±0.20	71.64±0.17	ND	7.57±0.15	3.74±0.13	ND	1.09±0.02	1.42±0.82	2.57±0.21
		8		0.84±0.04	1.13±0.05	11.73±0.52	68.72±0.85	2.13±0.14	7.98±0.20	3.44±0.09	0.20±0.01	0.74±0.09	1.01±0.17	2.10±0.27
		12		0.79±0.03	5.56±0.23	13.97±1.17	49.58±5.17	9.35±0.32	8.96±0.63	3.11±0.14	0.52±0.12	1.45±0.82	0.85±0.05	2.59±0.84
	48 h	0	0	0.97±0.01	ND	10.54±0.03	71.98±0.24	ND	7.64±0.09	4.51±0.06	ND	1.21±0.05	0.89±0.21	2.26±0.15
		8		0.94±0.01	1.26±0.04	11.57±0.16	67.91±0.67	2.06±0.08	8.16±0.19	4.03±0.08	0.12±0.01	0.82±0.03	0.71±0.02	2.43±0.11
		12		0.85±0.05	4.76±0.07	14.63±0.21	54.70±1.01	7.48±0.27	8.89±0.22	3.54±0.12	0.38±0.10	1.41±0.46	0.94±0.07	2.26±0.22
ML103 (pXZ18, pBAD33)	24 h	0	0	ND	ND	ND	ND	ND	ND	26.47±0.46	ND	23.29±0.30	36.37±0.53	13.87±0.99
		8		ND	ND	ND	ND	ND	ND	34.18±0.66	2.36±0.12	29.15±0.74	23.89±0.66	10.44±0.38
		12		ND	ND	ND	ND	0.15±0.08	ND	29.67±0.08	3.80±0.96	27.45±0.64	24.57±0.56	14.39±0.18
	48 h	0	0	ND	ND	ND	ND	ND	ND	25.85±0.35	ND	22.50±0.59	36.56±0.39	15.10±1.02
		8		ND	ND	ND	ND	ND	ND	33.11±0.09	2.25±0.09	30.05±0.94	24.66±0.82	9.94±0.38
		12		ND	ND	ND	ND	0.16±0.02	ND	29.96±0.43	3.52±0.57	28.36±0.38	24.09±0.70	13.90±0.18
ML103 (pXZM12, pBAD-*prpE*)	24 h	8	5	0.73±0.04	3.58±0.18	12.29±0.42	60.85±0.56	6.89±0.11	8.35±0.10	3.43±0.02	0.34±0.11	0.64±0.11	0.80±0.28	2.10±0.16
			10	0.76±0.03	4.69±0.02	12.09±0.27	58.28±0.32	8.87±0.22	7.95±0.18	3.29±0.09	0.44±0.01	0.74±0.01	0.86±0.17	2.02±0.18
			20	0.74±0.02	5.92±0.11	11.49±0.27	55.70±0.01	10.92±0.18	7.68±0.18	3.23±0.01	0.61±0.03	0.68±0.05	0.97±0.02	2.06±0.11
		12	10	0.48±0.10	9.14±0.12	11.85±0.17	45.42±0.32	16.86±0.18	8.33±0.09	3.16±0.07	0.94±0.08	0.87±0.01	1.04±0.11	1.92±0.05
			20	0.59±0.05	9.13±0.05	11.17±0.00	45.76±0.14	16.79±0.10	8.08±0.03	3.27±0.14	0.90±0.04	0.99±0.14	1.01±0.26	2.30±0.42
			50	0.62±0.09	7.66±0.07	10.51±0.08	50.09±0.22	14.00±0.38	8.02±0.06	3.47±0.03	0.72±0.05	1.07±0.06	1.12±0.25	2.73±0.25
	48 h	8	5	0.77±0.01	3.32±0.02	12.21±0.07	61.80±0.13	5.48±0.05	8.35±0.19	3.86±0.02	0.26±0.03	0.90±0.02	0.64±0.01	2.40±0.14
			10	0.76±0.02	4.13±0.11	12.08±0.20	59.69±0.47	7.00±0.25	8.18±0.10	3.74±0.04	0.36±0.01	0.89±0.03	0.68±0.00	2.49±0.04
			20	0.71±0.01	4.95±0.05	11.56±0.31	57.72±0.05	8.86±0.29	8.05±0.29	3.75±0.01	0.45±0.02	0.87±0.00	0.71±0.06	2.36±0.16
		12	10	0.62±0.01	7.84±0.08	12.05±0.16	47.59±0.43	14.85±0.19	8.77±0.04	3.53±0.04	0.75±0.10	1.08±0.02	0.65±0.03	2.27±0.16
			20	0.63±0.01	7.73±0.02	11.24±0.02	48.87±0.19	14.63±0.25	8.45±0.05	3.55±0.11	0.80±0.03	0.99±0.02	0.64±0.03	2.46±0.07
			50	0.63±0.01	6.48±0.04	10.16±0.05	53.17±0.29	11.91±0.09	7.98±0.18	3.88±0.03	0.69±0.02	1.09±0.03	1.10±0.10	2.92±0.14
ML103 (pXZ18, pBAD-*prpE*)	24 h	8	5	ND	ND	ND	ND	0.25±0.01	ND	31.91±3.58	5.10±0.64	27.26±2.80	21.46±2.35	14.01±7.83
			10	ND	ND	ND	ND	0.17±0.02	ND	31.64±0.32	5.02±0.24	26.95±1.12	23.82±0.22	12.41±0.76
			20	ND	ND	ND	ND	0.20±0.04	ND	31.38±0.32	6.29±0.25	27.63±0.28	23.15±0.37	11.36±0.32
		12	10	ND	ND	ND	ND	0.34±0.09	ND	31.72±0.79	7.97±0.49	29.42±0.21	21.35±1.06	9.20±0.40
			20	ND	ND	ND	ND	0.52±0.08	ND	31.56±0.32	11.65±0.97	27.94±0.44	20.05±0.43	8.28±0.42
			50	ND	ND	ND	ND	0.68±0.11	ND	31.48±0.74	12.88±0.69	26.69±0.31	20.40±0.55	7.87±0.79
	48 h	8	5	ND	ND	ND	ND	0.27±0.08	ND	33.13±1.31	6.64±1.74	29.36±0.90	21.15±1.22	9.44±0.81
			10	ND	ND	ND	ND	0.30±0.06	ND	33.01±0.17	6.61±0.57	28.43±0.21	21.55±0.69	10.11±0.29
			20	ND	ND	ND	ND	0.38±0.03	ND	32.85±0.54	8.81±0.68	28.04±0.19	20.58±0.38	9.33±0.21
		12	10	ND	ND	ND	ND	0.98±0.14	ND	32.20±0.23	12.37±0.91	28.23±0.24	18.74±0.66	7.48±0.44
			20	ND	ND	ND	ND	1.19±0.13	ND	32.40±0.29	16.49±1.30	27.15±0.60	16.77±0.40	6.00±0.47
			50	ND	ND	ND	ND	1.27±0.19	ND	33.66±0.68	15.39±1.94	26.86±0.71	17.35±0.42	5.48±0.47

ND not detected

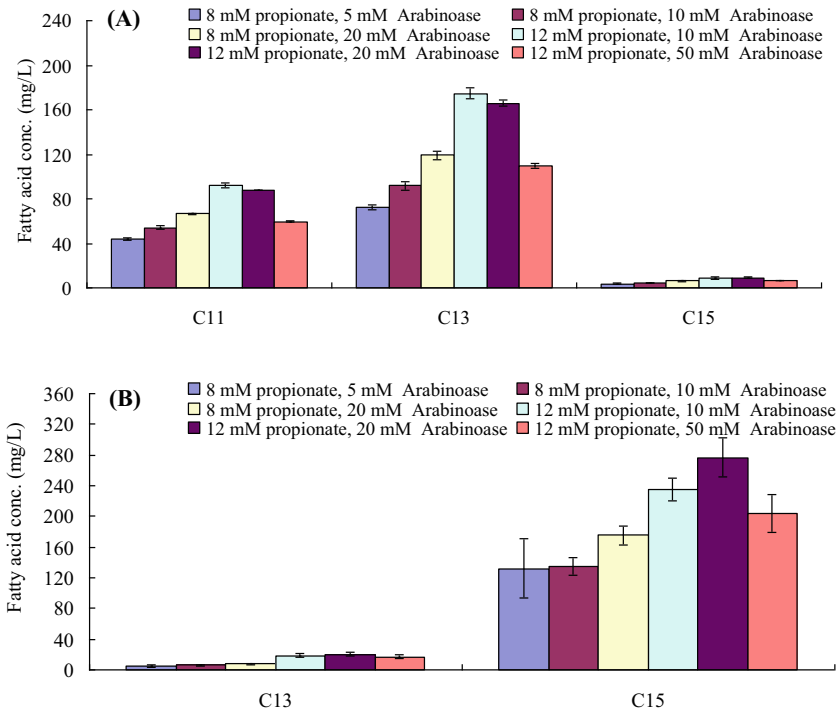

Fig. 4 Effects of *prpE* over expression combined with propionate addition on odd straight chain FFAs distribution in ML103 (pXZM12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*) at 48 h. **a** ML103 (pXZM12, pBAD-*prpE*); **b** ML103 (pXZ18, pBAD-*prpE*)

dodecanoic acid and dodecenoic acid decreased while odd straight chain FFAs increased. The highest percentages of undecanoic acid and tridecanoic acid reached 9.14 % and 16.86 % at 24 h with 12 mM of propionate and 10 mM arabinose. Both the percentage of odd straight chain FFAs, undecanoic acid and tridecanoic acid, dropped by about 15 % at 48 h due to further production of even chain FFAs. For the strain ML103 (pXZ18, pBAD-*prpE*), the percentages of tridecanoic acid and pentadecanoic acid increased with time and reached 1.19 % and 16.79 %, respectively. The odd straight chain pentadecanoic acid was the major odd straight chain FFAs, about 93.4 % of the total odd straight chain FFAs. The even chain FFAs tetradecanoic acid and hexadecenoic acid showed less change than those of hexadecanoic acid and octadecenoic acid.

Discussion

In this study, microbial biosynthesis of odd straight medium chain FFAs was achieved by the supplementation of extracellular propionate to metabolically engineered efficient even chain FFAs production *E. coli* strains. The strain carrying the acyl-ACP TE genes from *U. californica* produced undecanoic acid and tridecanoic acid as the major odd straight chain FFAs while the strains carrying the acyl-ACP TE genes from *R. communis* preferred pentadecanoic acid. Introducing the propionyl-CoA synthetase gene (*prpE*) from *S. enterica* into these strains has been shown to further improve the odd straight chain FFA production by increasing the intracellular propionyl-CoA availability.

Dellomonaco et al. (2011) showed that the stains with engineered reversal of the β-oxidation cycle produced C7, C9 and C11 fatty alcohols with small amount of even chain when propionate was added in the medium. In this study, the acyl-ACP TEs released FFAs from odd straight chain acyl-ACPs, and the odd straight medium chain FFAs were synthesized though the native FFAs synthesis cycle. Although further introducing the propionyl-CoA synthetase into these engineered strains enhanced the odd straight chain FFA production, the even straight chain FFAs still were the major products (Table 2). The initial and elongation step in the reversal of the β-oxidation cycle pathway is catalyzed by FadA, while the native FFAs synthesis pathway is catalyzed by KAS III. It seemed that the substrate specificity of propionyl-CoA of native KAS III in *E. coli* was much lower than that of the native FadA, and the Km for propionyl-CoA of FadA was also much lower than that of acetyl-CoA.

The native KAS III, which catalyzes the first step in fatty acid elongation cycle, prefers the acetyl-CoA as a substrate (Tsay et al. 1992). While Qiu et al. (2005) suggested that there was no detected activity of the native KAS III on propionyl-CoA. The measured in vitro specific activity of the native KAS III by Choi et al. (2000) indicated that it was most active with acetyl-CoA, and the specific activity on propionyl-CoA was about one-fourth of that on acetyl-CoA. The result of increased odd straight chain FFAs provided the in vivo

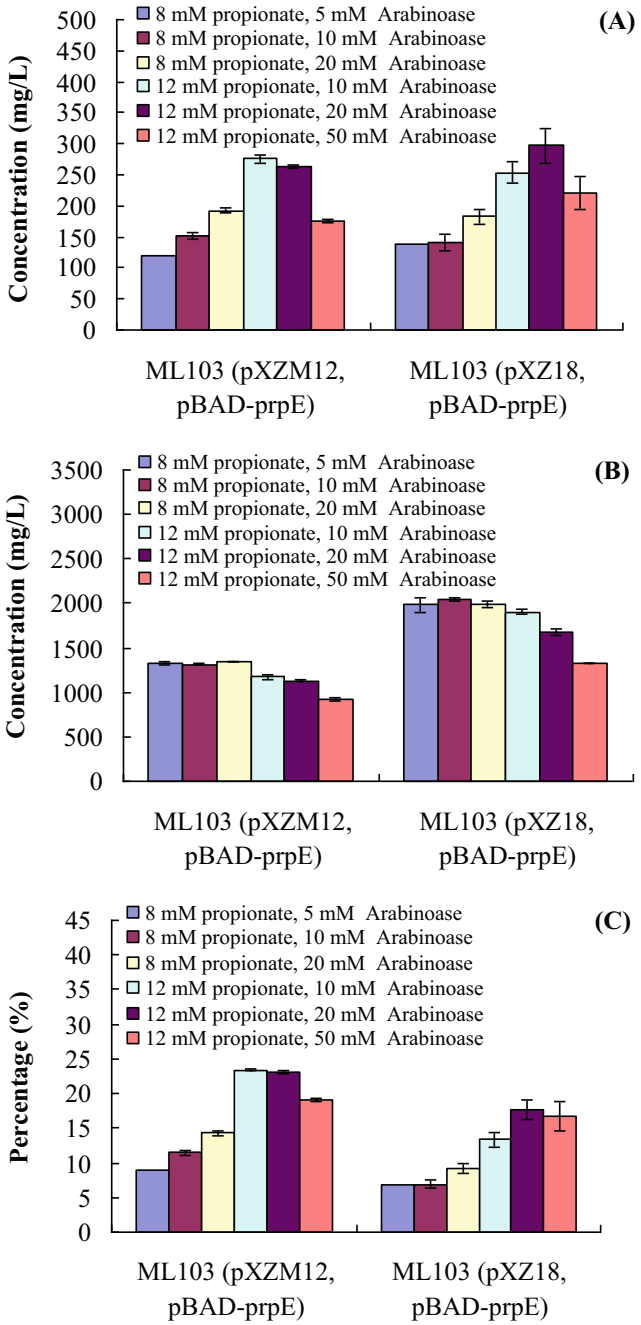

Fig. 5 Effects of *prpE* over expression combined with propionate addition on total odd straight chain FFAs and total FFAs produced by ML103 (pXZM12, pBAD-*prpE*) and ML103 (pXZ18, pBAD-*prpE*) at 48 h. **a** The total odd chain FFAs; **b** the total FFAs; **c** the percentage of total odd FFAs

evidence that the native KAS III has the ability to use propionyl-CoA as a substrate in addition to acetyl-CoA. The source of propionate in this study was provided extracellularly by adding it into the medium, and the formation of propionyl-CoA was catalyzed by propionyl-CoA synthetase. There are many different pathways which can synthesize proptionyl-CoA from glucose or glycerol, such as the citramalate/2-oxobutyrate pathway, the aspartate/2-oxobutyrate pathway, the methylmalonyl–CoA pathway, and a novel 3-hydroxypropionate pathway (Han et al. 2013; Reisch et al. 2013). Further studies will be needed to gain insight into the efficiency of the production of odd straight chain acyl-ACPs and synthesis of intracellular propionyl-CoA from carbon source directly. Meanwhile, using different KAS IIIs with higher specific activity for propionyl-CoA to further increase the concentration, yield and ratio of the odd straight chain FFAs is underway in this laboratory.

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