Open access • Journal Article • DOI:10.1007/S00253-013-5027-2

Fatty alcohol production in engineered E. coli expressing Marinobacter fatty acyl-CoA reductases — Source link

Aiqiu Liu, Aiqiu Liu, Xiaoming Tan, Lun Yao ...+1 more authors

Institutions: Chinese Academy of Sciences, Shandong University

Published on: 23 Jun 2013 - Applied Microbiology and Biotechnology (Springer Berlin Heidelberg)

Topics: Marinobacter, Fatty alcohol, Acyl-CoA and Escherichia coli

Appl Microbiol Biotechnol (2013) 97:7061–7071
DOI 10.1007/s00253-013-5027-2

BIOENERGY AND BIOFUELS

Fatty alcohol production in engineered *E. coli* expressing *Marinobacter* fatty acyl-CoA reductases

Aiqiu Liu · Xiaoming Tan · Lun Yao · Xuefeng Lu

Received: 24 February 2013 / Revised: 21 May 2013 / Accepted: 30 May 2013 / Published online: 23 June 2013

Abstract Although successful production of fatty alcohols in metabolically engineered *Escherichia coli* with heterologous expression of fatty acyl-CoA reductase has been reported, low biosynthetic efficiency is still a hurdle to be overcome. In this study, we examined the characteristics of two fatty acyl-CoA reductases encoded by Maqu_2220 and Maqu_2507 genes from *Marinobacter aquaeolei* VT8 on fatty alcohol production in *E. coli*. Fatty alcohols with diversified carbon chain length were obtained by co-expressing Maqu_2220 with different carbon chain length-specific acyl-ACP thioesterases. Both fatty acyl-CoA reductases displayed broad substrate specificities for C12–C18 fatty acyl chains in vivo. The optimized mutant strain of *E. coli* carrying the modified *tesA* gene and *fadD* gene from *E. coli* and Maqu_2220 gene from *Marinobacter aquaeolei* VT8 produced fatty alcohols at a remarkable level of 1.725 g/L under the fermentation condition.

Keywords *Escherichia coli* · Fatty alcohol · Fatty acyl-CoA reductase · *Marinobacter aquaeolei* VT8

Electronic supplementary material The online version of this article (doi:10.1007/s00253-013-5027-2) contains supplementary material, which is available to authorized users.

A. Liu · X. Tan · L. Yao · X. Lu (✉)
Key Laboratory of Biofuels, Shandong Provincial Key Laboratory of Energy Genetics, Qingdao Institute of Bioenergy and Bioprocess Technology, Chinese Academy of Sciences, No. 189 Songling Road, Qingdao 266101, China
e-mail: lvxf@qibebt.ac.cn

A. Liu
Ocean College, Shandong University (Weihai), Weihai 264209, China

A. Liu · L. Yao
University of Chinese Academy of Sciences, Beijing 100049, China

Introduction

Fatty alcohols are widely existing in many organisms including bacteria (Reiser and Somerville 1997; Wahlen et al. 2009), plants (Domergue et al. 2010; Metz et al. 2000; Rowland et al. 2006; Vioque and Kolattukudy 1997), mammals (Cheng 2004), etc. They are biologically synthesized as components of plant cuticle or precursors of wax esters that function as energy storage compounds in bacteria (Willis et al. 2011). Fatty alcohols are important raw materials and consumer products, and can be widely used in chemical industry such as plastics, detergents, lubricants, pharmaceuticals, and cosmetics (Gervajio 2005).

In industrial scale, fatty alcohols are chemically produced by hydrogenation of pretreated plant oil and animal fats, hydrolysis or reduction of wax esters, or catalytic oligomerization of ethylene (Carlsson et al. 2011). Recently microbial production of fatty acid-derived chemicals, such as fatty acid esters, fatty alcohols, and fatty alkanes, from renewable carbon sources, is drawing more and more attentions from both academy and industry (Fortman et al. 2008; Kalscheuer et al. 2006; Peralta-Yahya et al. 2012). In nature, fatty alcohols are synthesized de novo by reducing fatty acyl-CoA (Coenzyme A) (or ACP (Acyl carrier protein)) intermediates obtained *via* the fatty acid biosynthetic pathway (Fig. 1). In this process, thioesterase (TE), functioning in hydrolysis of fatty acyl-ACP at the appropriate chain length to produce free fatty acids, plays a key role for overproduction of fatty acids by releasing feedback inhibition of fatty acid biosynthesis caused by fatty acyl-ACPs (Cho and Cronan 1995; Davis 2000; Jiang and Cronan 1994). Recently, by using the fatty acyl-ACP thioesterases with different chain-length substrate specificities, such as *tesA* gene from *Esccherichia coli* (C16:0), CCTE gene from *Cinnamomum camphorum* (C14:0), and BTE gene from *Umbellularia californica* (C12:0), the fatty acid synthesis pathway has been exploited for the overproduction of free fatty acids in microbes (Liu et al. 2010; Liu and Curtiss 2011; Lu et al. 2008; Zhang et al. 2012). And to generate the fatty acid-derived chemicals, fatty acid activating enzyme is also necessary to

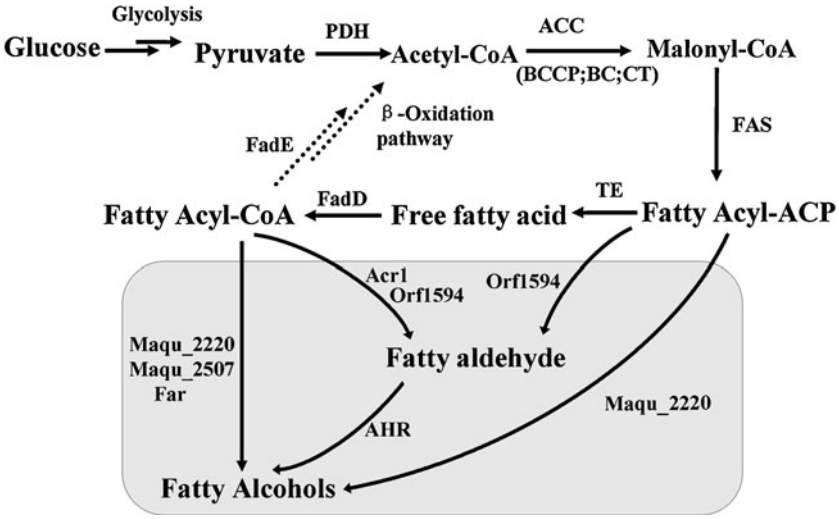

Fig. 1 Biosynthetic pathways of fatty alcohols in genetically engineered *E. coli*. The *shadow part* indicates the engineered pathways for the fatty alcohol synthesis in this work. *PDH* pyruvate dehydrogenase, *ACC* acetyl-CoA carboxylase, *BCCP* biotin carboxyl carrier protein, *BC* biotin carboxylase, *CT* carboxyltransferase a, b two subunits, *FAS* fatty acid synthase, *TE* thioesterases, *ACP* acyl carrier protein, *AHR* aldehyde reductase, *FadD* fatty acyl-CoA synthase, *FadE* acyl-CoA dehydrogenase, *Far* fatty acyl-CoA reductase

activate the fatty acid precursor to fatty acyl-CoA or fatty acyl-ACP by fatty acyl-CoA synthetase (FadD; Kameda and Nunn 1981) or fatty acyl-ACP synthetase (AAS; Kaczmarzyk and Fulda 2010). Reduction of the fatty acyl-CoA to the corresponding fatty alcohol is catalyzed by fatty acyl-CoA reductase (FAR). A variety of fatty acyl-CoA reductases from jojoba (Metz et al. 2000), *Arabidopsis thaliana* (Doan et al. 2009) and mouse(Cheng 2004), have been identified and characterized, respectively, and some of them have been overexpressed for the production of fatty alcohols in genetically engineered *E. coli*. Steen et al. firstly combined heterologous expression of FAR derived from *Acinetobacter calcoaceticus* BD413 and fatty acid overproduction in order to construct an fatty alcohol producing strain of *E. coli* with a yield of 60 mg/L (Steen et al. 2010). Another group reported the high-specificity production of both C12/14 (75.0 % of the total fatty alcohol production) and C16/18 alcohols (89.2 % of the total fatty alcohol production) in engineered *E. coli* by expressing different thioesterases and FAR with different substrate specificity (Zheng et al. 2012). Fatty alcohols were also produced by the mutant strain of photosynthetic *Synechocystis* sp. PCC 6803 with overexpressing FAR gene from jojoba (Tan et al. 2011). Though fatty alcohols have been produced in these engineered microorganisms, the production of fatty alcohols was relatively too low to be economically feasible for industrial applications. Previous study suggested that FAR activity might be one of limiting factors for fatty alcohol production (Steen et al. 2010). Thus the discovery and expression of more FAR enzymes with higher efficiency should be a promising strategy for enhancing fatty alcohol production in genetically engineered microorganisms.

A recent report identified a fatty acyl-ACP reductase (Orf1594) from the cyanobacterium *Synechococcus elongatus* PCC 7942 that catalyzes the reduction of fatty acyl-ACP or fatty acyl-CoA to fatty aldehyde but prefer the former (Schirmer et al. 2010). This fatty acyl-ACP reductase was also expressed in *E. coli* harboring endogenous aldehyde reductases for producing fatty alcohols (Schirmer et al. 2010). Wahlen et al. characterized a novel fatty aldehyde reductase (Maqu_2220) from *Marinobacter aquaeolei* VT8 which was found to be able to reduce fatty aldehydes to fatty alcohols (Wahlen et al. 2009). However, Hofvander et al. further confirmed that the same enzyme possesses the ability to catalyze not only fatty aldehydes but also acyl-CoA or acyl-ACP to corresponding fatty alcohols (Hofvander et al. 2011). Recently, another fatty acyl-CoA reductase (Maqu_2507), which catalyzes the reduction of fatty acyl-CoA directly to the corresponding fatty alcohol, was identified from *M. aquaeolei* VT8 too (Willis et al. 2011). The in vitro enzymatic characterizations showed that these two fatty acyl-CoA reductases, Maqu_2220 and Maqu_2507, demonstrated higher activity and wider range of substrate specificity in comparison to the other FAR enzymes previously characterized (Metz et al. 2000; Reiser and Somerville 1997; Teerawanichpan and Qiu 2010; Teerawanichpan et al. 2010), which was displaying their potentials to be good candidate catalysts for fatty alcohol production in engineered *E. coli*.

In this work, we evaluated fatty alcohol production capacity in *E. coli* by using two fatty acyl-CoA reductases encoded by *maqu_2507* and *maqu_2220* from *M. aquaeolei* VT8, which was compared with another two reported FARs encoded by *orf1594* from *S. elongatus* PCC 7942 and *acr1* from *Acinetobacter* sp. M-1. The effects of overproduction of fatty acids and overexpression of thioesterases with different substrate specificity on fatty alcohol production in recombinant strain of *E. coli* carrying these two FAR genes from *M. aquaeolei* VT8 were analyzed. The optimized mutant strain of *E. coli* carrying *'tesA*, *fadD* and Maqu_2220 gene produced fatty alcohols at a remarkable level of 1.725 g/L under the fermentation condition. This work shows the promising potential of two fatty acyl-CoA reductases encoded by *maqu_2507* and *maqu_2220* from *M. aquaeolei* VT8 as reducing enzymes for fatty alcohol production.

Materials and methods

Chemicals and reagents

1-Pentadecanol was obtained from Sigma-Aldrich (USA). Other chemicals were from Merck (Germany) or Ameresco (USA). Oligonucleotides and codon optimized genes were synthesized by Sangon (Shanghai, China). Taq DNA polymerases, Pfu DNA polymerase, T4 DNA ligase, and all restriction endonucleases were from Fermentas (Burlington, Canada) or Takara (Japan). The kits used for molecular cloning were from Omega (Norcross, USA) or Takara (Kyoto, Japan).

Strains and plasmids construction

The recombinant plasmids and strains used in this study are listed in Table 1. All oligonucleotide primers used in this study are listed in Table 2. *E. coli* DH5α was used for all plasmids construction. *E. coli* BL21(DE3) and *E. coli* BL21 (Δ*fadE*) (Duan et al. 2011) were used as host cells for fatty alcohol production. The strain *M. aquaeolei* VT8 (ATCC 700491) was bought form DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH). The expression vector pET-21b and PET-28b were purchased from Novagen (Madison, WI, USA). The expression vector pBAD33 was kindly provided by Professor Jonathan Beckwith at Harvard Medical School.

For building the biosynthesis pathway of fatty alcohols in *E. coli*, recombinant plasmids with four different FARs were constructed as follows. The Maqu_2507 gene (GenBank: YP_959769.1) and Maqu_2220 gene (GenBank: YP_959486) from *M. aquaeolei* VT8 were separately obtained by polymerase chain reaction (PCR) using primers Mq2507-1/2 and Faldr-1/2 (Table 2), respectively, and cloned into pET-28b vector between *Nde*I and *Xho*I sites, creating plasmids pXT109 and pAL136, respectively. The *orf1594* gene (GenBank accession no. 3775018) was amplified by PCR from the genomic DNA of *S. elongatus* PCC 7942 with primer orf1594-1/orf1594-2, and then sub-cloned into the *Nde*I and *Xho*I sites of pET-21b vector, creating plasmid pAL130. The *acr1* gene of *Acinetobacter* sp. M-1 was codon optimized, chemically synthesized (GenBank accession no. KC6929988), and then cloned into pET-28b vector between *Nde*I and *Bam*HI sites, creating plasmid pAL134. To co-express *fadD* and FAR in the same plasmid, the *Xba*I–*Xho*I double-digested DNA fragments of pXT109 and pAL136 were separately cloned into the *Spe*I and *Xho*I sites of pXT3, resulting in plasmids pAL144 and pAL145, respectively.

Plasmids with different thioesterases were constructed as follows. The *Xba*I–*Spe*I double-digested DNA fragment of pXL49 (Lu et al. 2008) was inserted into the *Xba*I site of pBAD33, creating plasmid pKC7 for expression of the thioesterase from *C. camphorum* (CCTE). BTE gene (without leading sequence) (GenBank accession no. KC6929989) of *U. californica* was amplified from pSai328 plasmid with primers BTE-R/BTE-F and cloned into pKC7 resulting pAL143. The *E. coli 'tesA* gene (without leading sequence) was excised by *Bam*HI and *Hin*dIII from pMSD15 (Davis 2000) and cloned into the *Bam*HI and *Xba*I sites of pKC7, resulting in pKC10. In this process, both *Hin*dIII and *Xba*I sites were blunted by T4 DNA polymerase before digestion by *Bam*HI.

The *E. coli fadD* gene was excised from pXT3 (Duan et al. 2011) via digestion with *Bgl*II/*Xho*I and cloned into pBAD33 digested by *BamH*I and *Sal*I, resulting in pAL125. For construction of another plasmid with the *E. coli fadD* gene, an ampicillin gene and a pSC101 origin, a fragment including P_{BAD} promoter and multiple clone sites and *rrnB* T1/T2 (terminator) was amplified from pBAD33 with primer PBAD33-F/R and cloned into pFN476 digested by *Bam*HI resulting pKC17. The *Nde*I–*Xho*I double-digested DNA fragment of pXT3 was inserted into pKC7 by *Nde*I and *Sal*I resulting pKC18. And then, the *E. coli fadD* gene was excised from pKC18 by *Kpn*I and *Hin*dIII, and inserted into the same sites of pKC17, resulting in pKC11. The fidelity of the inserts was reconfirmed based on DNA sequencing results.

Recombinant strains of *E. coli* were constructed by transformation of *E. coli* BL21 (DE3) or BL21 (Δ*fadE*) competent cells with corresponding plasmids (Table 1).

Inoculum preparation and shake flask cultures

A single colony from a freshly transformed plate was grown for about 12 h at 30 °C in 5 mL of LB medium supplemented with 50 mg/L ampicillin or 25 mg/L kanamycin or 17 mg/L chloramphenicol if necessary. The culture was then inoculated into a 250-mL flask containing 50 mL modified mineral medium at a final OD_{600} of 0.05 and were grown at 30 °C. The modified mineral medium has the following composition (Schirmer et al. 2010): 6 g/L Na_2HPO_4, 3 g/L KH_2PO_4, 0.5 g/L NaCl, 2 g/L NH_4Cl, 0.25 g/L $MgSO_4{\cdot}7\ H_2O$, 2 mg/L $ZnCl_2{\cdot}4\ H_2O$, 27 mg/L $FeCl_3{\cdot}6\ H_2O$, 2 mg/L $Na_2MoO_4{\cdot}2\ H_2O$, 11 mg/L $CaCl_2$, 0.5 mg/L H_3BO_3, 1.9 mg/L $CuSO_4{\cdot}5\ H_2O$, 1 mg/L thiamine, 200 mM Bis–Tris (pH 7.25), and 0.1 % (*v*/*v*) Triton-X100. Three percent glucose was added as carbon source and antibiotics were used for plasmid maintenance at the following concentrations: 50 mg/L ampicillin or 25 mg/L kanamycin or 17 mg/L chloramphenicol. The genes driven by P_{BAD} promoter were induced with 0.4 % L-arabionse when OD_{600} reached 0.6–0.8, whereas the genes driven by P_{T7} promoter were induced by 0.5 mM IPTG 1 h later. At about 36 h after induction by IPTG (Isopropyl β-D-thiogalactoside), cell cultures were extracted for GC-MS (gas chromatography with detection by mass spectrometry analysis).

Fermentation

The batch fermentation was performed in a 5-L fermenter (Biostat Bplus, Sartorius) using a 3-L working volume, at

30 °C and in the same medium as the flask fermentation except the lack of Bis-tris and Triton-X100. During the fermentation process, the pH was controlled at 7.2 by automatic addition of 2 M HCl or 5 M NaOH. The stirring speed was first set at 200 rpm (rotation per minute) and then associated with the dissolved oxygen (DO) to maintain a DO concentration of above 20 % saturation. The flow rate of air was maintained at 2 L/min. When the cells of AL322

Table 1 Plasmids, strains constructed and used in this study

Plasmids or strains	Relevant characteristic(s)	Reference or source
pET-21b	Ap^r; pBR322 ori; *lacI*; T7 promoter	Novagen
pET-28b	Kan^r; pBR322 ori; *lacI*; T7 promoter	Novagen
pBAD33	Cm^r; p15A ori; P_{BAD} promoter	Gift from Jonathan Beckwith
pAL130	Ap^r; PET-21b derivative containing *orf1594* from *Synechococcus elongatus* PCC 7942; T7 promoter	This study
pAL134	Kan^r; pET-28b derivative containing *acr1* gene from *Acinetobacter* sp. M; T7 promoter	This study
pAL136	Kan^r; pET-28b derivative containing *maqu_2507*; T7 promoter	This study
pXT109	Kan^r; pET-28b derivative containing *maqu_2220*; T7 promoter	This study
pAL125	Cm^r; pBAD33 derivative containing *E. coli fadD*; T7 promoter	This study
pAL143	Cm^r; pBAD33 derivative containing CCTE gene from *Cinnamomum camphorum* and BTE gene from *Umbellularia californica*; P_{BAD} promoter	
pAL144	Kan^r; pET-28b derivative containing *fadD* and *maqu_2507*; T7 promoter	This study
pAL145	Kan^r; pET-28b derivative containing *fadD* and *maqu_2220*; T7 promoter	This study
pMSD8	Ap^r; pFN476 derivative containing *E. coli accBCDA* genes; T7 promoter	(Davis 2000)
pMSD15	Cm^r; pACYC184 derivative containing *E. coli 'tesA* gene (without leading sequence); P_{BAD} promoter	(Davis 2000)
pKC7	Cm^r; pBAD33 derivative containing CCTE gene ; P_{BAD} promoter	This study
pKC10	Cm^r; pBAD33 derivative containing *E. coli 'tesA* gene and CCTE gene; P_{BAD} promoter	This study
pKC11	Ap^r; pFN476 derivative containing *E. coli fadD*; P_{BAD} promoter	This study
pKC17	Ap^r; pFN476 derivative containing fragments including P_{BAD} promoter and *rrn*B T1/T2 (terminators) from pBAD33; P_{BAD} promoter	This study
pKC18	Cm^r; pBAD33 derivative containing *E. coli fadD*; P_{BAD} promoter	This study
pSai328	Ap^r; containing BTE gene from *Umbellularia californica*; trc promoter	Gift from Xinyao Liu
pXT3	Kan^r; pET-28b derivative containing *E. coli fadD*; T7 promoter	(Duan et al. 2011)
Strains		
DH5α	F^-, *gyrA*96, *deoR phoA*, *supE*44 , *relA*1, Φ80d/*lacZ*ΔM15, Δ(*lacZYA-argF*)U169, *hsdR*17 ($rK^- mK^+$) λ^-, *endA*1, *thi*-1, *recA*1	Takara
BL21(DE3)	F^- *ompT hsdSB*(*r*B- *m*B-) *gal* (λ *c I* 857 *ind*1 *Sam*7 *nin*5 lacUV5-T7 gene1) *dcm*(DE3)	Novagen
BL21(Δ*fadE*)	BL21 (DE3) knocking out *fadE* gene	(Duan et al. 2011)
AL304	BL21(Δ*fadE*) bearing pAL130 and pAL125	This study
AL305	BL21(Δ*fadE*) bearing pAL134 and pAL125	This study
AL306	BL21(Δ*fadE*) bearing pAL136 and pAL125	This study
AL307	BL21(Δ*fadE*) bearing pXT109 and pAL125	This study
AL338	BL21(Δ*fadE*) bearing pAL144	This study
AL339	BL21(Δ*fadE*) bearing pAL144 and pAL143	This study
AL340	BL21(Δ*fadE*) bearing pAL144, pAL143 and pMSD8	This study
AL379	BL21(Δ*fadE*) bearing pAL145	This study
AL346	BL21(Δ*fadE*) bearing pAL145 and pAL143	This study
AL378	BL21(Δ*fadE*) bearing pAL145and pKC7	This study
AL362	BL21(Δ*fadE*) bearing pAL145and pMSD15	This study
AL363	BL21(Δ*fadE*) bearing pAL145 and pKC10	This study
AL321	BL21(Δ*fadE*) bearing pXT109	This study
AL312	BL21(Δ*fadE*) bearing pXT109 and pKC11	This study
AL322	BL21(Δ*fadE*) bearing pXT109, pKC11 and pMSD15	This study

ori origin, *Ap* ampicillin, *Kan* kanamycin, *Cm* chloramphenicol

Table 2 Primers used in this study

Primers	Sequence (5′ →3′)[a]
orf1594-1	AGACATATGCCGCAGCTTGAAGC
orf1594-2	TATACTCGAGAATTGCCAATGCCAAG
Mq2507-1	ACATATGAATTATTTCCTGACAGGCGGC
Mq2507-2	TCTCGAGTTACCAGTATATCCCCCGCATAAT
Faldr-1	TCATATGGCAATACAGCAGGTACATCA
Faldr-2	ACTCGAGTCAGGCAGCTTTTTTGCGC
BTE-F	GTCGACTCTAGAAGGAGGATTATAAAATGCTAGAGTGGAAGCCGAAACC
BTE-R	CTCGAGAAGCTTACACGCGCGGTTCGGC
PBAD33-F	CCATGGCCGCTTATTAAAAGCATTCTGTAAC
PBAD33-R	CCATGGGAAGCATTTATCAGGGTTATTGTCT

[a] Underlined nucleotides represent the restriction enzyme sites

were grown to an OD_{600} of about 10, L-arabinose was added to the media at a final concentration of 0.4 % for inducing the expression of *'tesA* gene and *fadD* gene. One hour later, 0.5 mM IPTG was added for inducing the expression of Maqu_2220. Aliquots of fermentation broth were collected at a series of time points and immediately kept at −80 °C for the further fatty alcohol analysis.

Analytic method

For fatty alcohol analysis, 4 mL culture was mixed thoroughly with 4 mL of organic solvent containing chloroform and methanol (the ratio of organic solvents is 2:1 by *v*/*v*), together with 0.04 mg 1-pentadecanol added as an internal standard. The organic phase was then collected and evaporated to dryness under nitrogen atmosphere, and re-dissolved in 1 mL of *n*-hexane. A 1-μL aliquot was analyzed by GC-FID (gas chromatography with detection by Flame ionization detector) using an Agilent 7890A system equipped with a HP-INNOWax (30-m×250 mm×0.25 mm). Nitrogen atmosphere (constant flow 1 mL/min) was used as the carrier gas. Product identities were also confirmed by GC-MS. The temperature of the injector was 250 °C and the following temperature program was applied: 100 °C for 1 min, increase of 5 °C/min to 200 °C then increase of 25 °C/min to 240 °C for 15 min. Authentic standards were used to quantify individual fatty alcohol.

Results

Fatty alcohol production in FAR-expressing *E. coli* strains with feeding of free fatty acids

To screen fatty acyl-CoA reductase with higher catalytic activity and broader substrate specificity for fatty alcohol production, four different fatty acyl-CoA reductase genes, *orf1594* from *S. elongatus* PCC 7942, *acr1* from *Acinetobacter* sp. M-1, *maqu_2220* and *maqu_2507* from *M. aquaeolei* VT8, were overexpressed under control of the strong P_{T7} promoter in an *E. coli* BL21 (DE3) mutant strain with overexpression of acyl-CoA synthetase (*fadD*) from *E. coli*. The resulting engineered strains were designated as AL304, AL305, AL306, and AL307, respectively. The in vivo conversion efficiencies and substrate specificities of these FARs were tested by culturing under shake flask condition in the presence of an equivalent blend (200 mM each) of lauratic acid (C12:0), myristic acid (C14:0), palmitic acid (C16:0), palmitoleic acid (C16:1), stearic acid (C18:0), and oleic acid (C18:1). As shown in Fig. 2a, a significant difference in fatty alcohol production was observed. In the presence of enough exogenous free fatty acids, *E. coli* strains expressing *orf1594* (AL304) or *acr1* (AL305) totally produced 20.7 mg/L or 69.5 mg/L fatty alcohols respectively, while the yields of fatty alcohols in AL306 (containing Maqu_2507 gene) and AL307 (containing Maqu_2220 gene) were 319.7 mg/L and 646.7 mg/L which were 15 and 31-fold higher than that of AL304, respectively. Since acyl-CoA ligase (*fadD*) from *E. coli* has broad substrate specificity towards C6 to C18 free fatty acids (Black et al. 1992; Kameda and Nunn 1981), the activity and substrate specificity of these FARs could be inferred from the fatty alcohols produced by the engineered strains. The above results indicate that the Maqu_2220 among the four candidate enzymes was relatively efficient for fatty alcohol production in *E. coli* with more stable expression or more efficient activity of this enzyme under the current experimental conditions.

As for the carbon chain length distribution of fatty alcohols displayed in Fig. 2b, AL304 predominantly produced C16 and C18 fatty alcohols, especially C16:0 and C18:1 products accounting for 89.3 % of total fatty alcohols. However, C12 and C14 fatty alcohols composed the majority of total fatty alcohols produced by AL305 and AL306. Compared to other engineered *E. coli* strains, the carbon chain length of fatty alcohols produced by AL307 ranges broadly from C12 to C18. In addition, all these four fatty acyl-CoA reductases exhibited poor specificity in vivo for C18 fatty acyl-CoA.

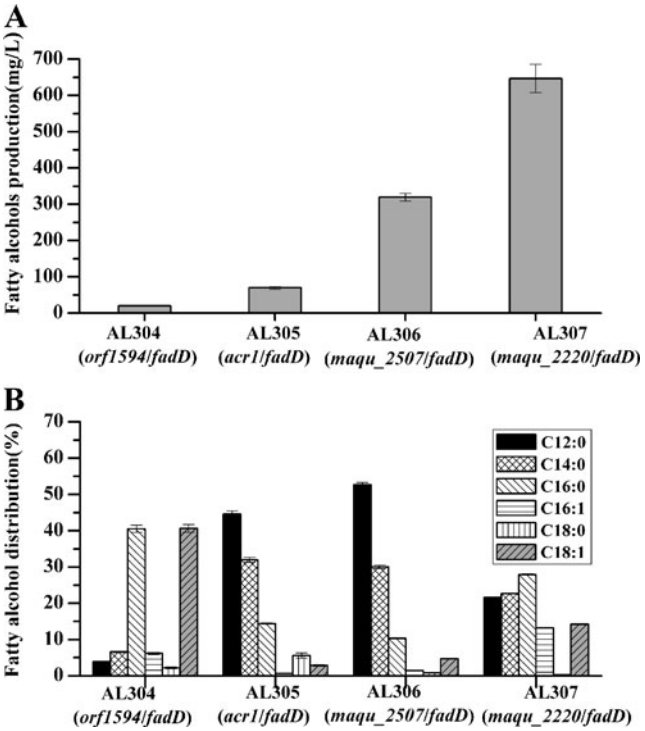

Fig. 2 Fatty alcohol production in different *E. coli* mutant strains by feeding an equivalent blend of C12–C18 even-carbon chain length free fatty acids into culture medium (**a**) and carbon chain length distribution of fatty alcohols in different recombinant strains with co-expressing *fadD* and *orf1594* (AL304) or *acr1* (AL305) or *maqu_2507* (AL306) or *maqu_2220* (AL307; **b**). *Values* and *error bars* represent the mean and standard deviation of triplicate experiments

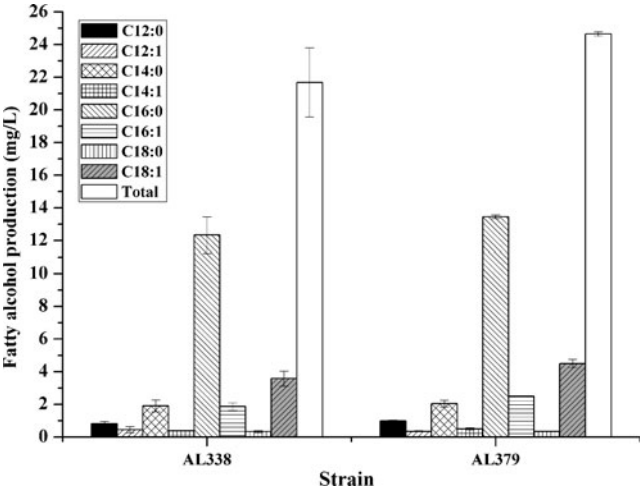

Fig. 3 De novo fatty alcohol production of AL338 (containing plasmid pAL144 with *fadD* gene and *maqu_2507* gene) and strain AL379 (containing pAL145 with *fadD* gene and *maqu_2220* gene) from glucose

De novo fatty alcohol production from glucose with expression of two fatty acyl-CoA reductases from *M. aquaeolei* VT8

To examine the efficiencies of each of two FARs from *M. aquaeolei* VT8 on fatty alcohol production from glucose respectively, *E. coli* BL21(Δ*fadE*) with the deletion of *fadE* gene, which can accumulate fatty acyl-CoA by interrupting the fatty acid degradation pathway, was used as the starting strain. The *fadD* gene together with Maqu_2507 or Maqu_2220 gene was co-expressed in *E. coli* BL21 (Δ*fadE*), resulting AL338 and AL379 mutant strains. As shown in Fig. 3, the fatty alcohol yields of AL338 and AL379 were 21.7 and 24.6 mg/L, respectively, which were much less than the productivities observed in the above exogenous fatty acid feeding experiment. The significant differences in fatty alcohol production between the two experiments indicate that the supply of free fatty acids might be the limited factor for the process of fatty alcohol production in these *E. coli* strains containing FAR gene from *M. aquaeolei* VT8. In addition, the composition of fatty alcohols produced in AL338 or AL379 is also obviously difference from that in the *E. coli* strain containing the corresponding FAR gene with feeding free fatty acids. The fatty alcohols with the longer carbon chain length (C16 and C18), accounting for 80.7 % and 90.1 % of total fatty alcohols, respectively, were the major constitutes in both AL338 and AL379. This might be because C16 and C18 fatty acyl chains dominate in *E. coli*.

Effect of overproduction of fatty acids on fatty alcohol accumulation in *E. coli* mutant strain with expression of Maqu_2507

To evaluate the effect of overproduction of fatty acids on fatty alcohol accumulation, the plasmid pAL143 containing both CCTE gene encoding thioesterase from *C. camphorum* and BTE gene encoding thioesterase from *U. californica*, was used to transform the *E. coli* mutant strain AL338 with overexpression of *fadD* and Maqu_2507 genes, generating AL339 mutant strain. Both pAL143 and pMSD8 (Davis 2000) with overexpression of *accBCDA* gene encoding acetyl-CoA carboxylase from *E. coli* was used to transform AL338 generating AL340 mutant strain. GC analysis of fatty alcohol production in AL338, AL339, and AL340 strains cultured under shake flask condition showed that the amount of fatty alcohols in AL339 was significantly increased to 218.2 mg/L, 9-fold higher than that of the control strain AL338 (Fig. 4). Furthermore, the percentage of medium-chain fatty alcohols (12:1, 12:0, 14:1, 14:0, and 16:1) in total fatty alcohols were dramatically increased, especially the C12 and C14 fatty alcohols, accounting for 89 % of total fatty alcohols. At the same time, the percentage of C16 and C18 fatty alcohols dropped drastically from more than 60 % to about 9 % and from more than 15 % to less than 2 %, respectively. These findings are consistent with the substrate specificity of CCTE and BTE (Voelker and Davies 1994; Yuan et al. 1995), which prefer C12 acyl-ACP and C14 acyl-

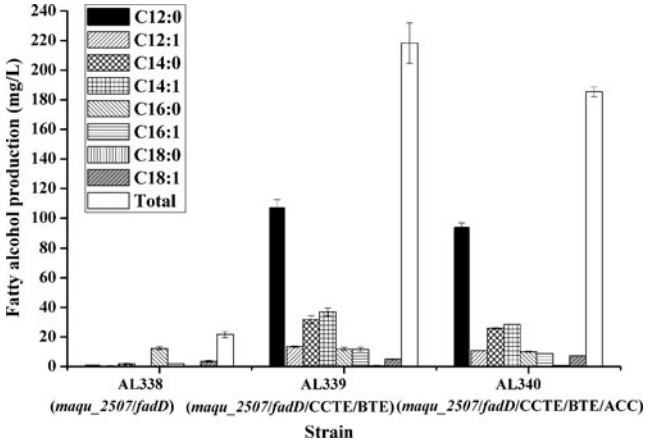

Fig. 4 Fatty alcohol production of engineered strains overexpressing Maqu_2507 gene combined with *fadD*, CCTE and BTE, and ACC genes

ACP, respectively. Unexpectedly, a further improvement in fatty alcohol production was not observed when acetyl-CoA carboxylase (ACC) was overexpressed in the strain AL340.

Effect of overexpression of thioesterases with different substrate specificity on fatty alcohol production in *E. coli* mutant strain with expression of Maqu_2220

To further examine effect of overexpression of different thioesterases on fatty alcohol production, AL379 strain that is *E. coli* BL21 (Δ*fadE*) carrying the plasmid pAL145 containing *fadD* and Maqu_2220 genes was used as the starting control strain. And different overexpression cocktail of thioesterases, which were *'tesA*, CCTE, CCTE and *'tesA*, and CCTE and BTE, were introduced into the control strain AL379, respectively, resulting in four new mutant strains AL362, AL378, AL363, and AL346. As shown in Fig. 5, different acyl-ACP thioesterases exhibited different effects on the quantities and the carbon chain length distributions of fatty alcohols. AL379 only produced less than 40 mg/L fatty alcohols with C16:0 and C18:1 as major components. Overexpression of *'tesA* or CCTE gene alone resulted in a 5.4- or 6.6-fold increase in the yield of fatty alcohols. And expression of CCTE together with BTE gene in AL346 led to a further increase in fatty alcohol production reaching 258.3 mg/L, which was 8-fold that of the control strain. Overexpression of *'tesA* increased the percentages of C14 fatty alcohol from 6 % to 36 %, while CCTE improved the percentages of C14 and C14:1 fatty alcohol from less than 7 % to about 27 % and from less than 2 % to more than 34 %, respectively. Overexpression of BTE further promoted the production of fatty alcohols with carbon chain length C12, and fatty alcohols with C12, C12:1, C14, and C14:1 carbon chain length, are the major components accounting for 86.6 % of total fatty alcohols in AL346. However, co-expression of *'tesA* and CCTE genes in AL363 led to an obvious decrease in fatty alcohol production, compared with the strain with expression of *'tesA* or CCTE genes alone.

Optimization of FAR expression system for improving fatty alcohol production

As described above, *fadD* gene together with Maqu_2220 gene were ligated together and placed under the control of P_{T7} promoter in the same plasmid pAL145 for fatty alcohol production in *E. coli* (Fig. S1). To optimize the expression system of Maqu_2220, another plasmid pKC11 was constructed only for the *fadD* expression, while pXT109 with Maqu_2220 gene driven by P_{T7} was only for the expression of Maqu_2220 gene (Fig. S1). Fatty alcohol production in strains AL321 (containing pXT109), AL312 (containing pXT109 and pKC11), and AL322 (containing pXT109, pKC11 and pMSD15) were measured by GC-MS or GC- and shown in Fig. 6. The titer of fatty alcohols produced in AL321 with only expression of Maqu_2220 was 253 mg/L and close to the best yield obtained from previously constructed AL346 with co-expression of Maqu_2220, *fadD*, CCTE, and BTE. Co-expression of *fadD* and *maqu_2220* in AL312 led to increased fatty alcohol levels to 379.3 mg/L, 50 % higher than that of AL321. Furthermore, when *'tesA* was co-expressed with *fadD* and *maqu_2220* in the AL322, the titer of fatty alcohol was 422 mg/L, with a composition dominated by C16 and C18 saturated and unsaturated fatty alcohols, constituting 82.5 % of total fatty alcohols.

Fatty alcohol production in the *E. coli* mutant strain AL322 under batch fermentation

To evaluate fatty alcohol production in a scalable process, the batch fermentation of AL322 was carried out in a 5-L fermenter with 3 L modified M9 medium and 30 g/L glucose as the initial culture medium. At an OD_{600} of ~10, the culture was induced with 0.4 % L-arabinose to express the *E. coli* thioesterase from plasmid pMSD15 and *fadD* from plasmid pKC11. One hour later, IPTG was added to a final concentration of 0.5 mM for inducing the expression of Maqu_2220. Thirty grams per liter glucose was added to the cultures for supplementing the carbon source 12 h after induction by IPTG. The maximum optical density of OD_{600} was about ~19 during fermentation. The production of fatty alcohol increased along with the cell growth over the course of fermentation, and reached a maximum of 1,725 mg/L after 110 h, which is about 3-fold than that in shake flasks. And the highest fatty alcohol productivity is 104.4 mg/L/OD. The glucose conversion efficiency was calculated to be 28.3 mg fatty alcohol/g glucose during all the course of fermentation.

The fatty alcohol compositions of AL322 under batch fermentation were shown in Fig. 7c. The pattern of fatty

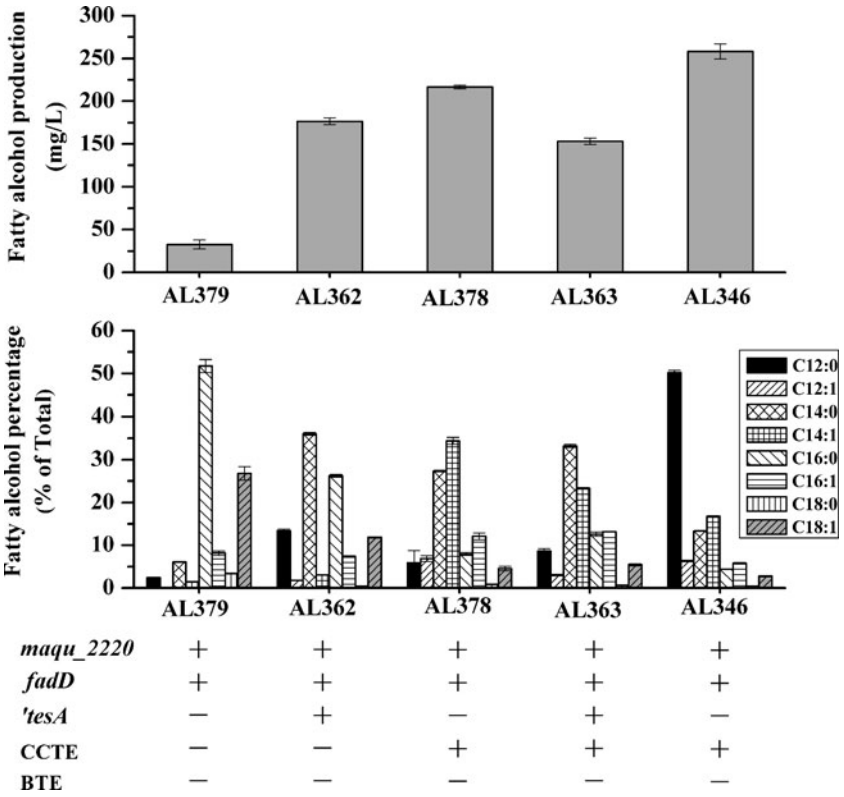

Fig. 5 Fatty alcohol production of engineered strains overexpressing *maqu_2220* combined with *fadD* gene and different carbon chain-specific fatty acyl-ACP thioesterases *'tesA* (AL362), CCTE (AL378), CCTE and *'tesA* (AL363), and CCTE and BTE (AL346; **a**); and the carbon chain length distribution of fatty alcohols in these engineered strains (**b**)

alcohol compositions changed with culture time. At the earlier 24 h sampling before induction, the target product mostly composed of C16 and C18 saturated and unsaturated fatty alcohols, with more C16 chains than C18. At the 72 h time point, the composition of fatty alcohol changed greatly. The percentage of C18:1 fatty alcohol dropped from more than 34.4 % at 24 h to around only 9.6 % while both C14 and C16:1 increased from about 9.4 % and 12.3 % to around 33.2 % and 24.8 %, respectively. As a result, the strain accumulated fatty alcohols with three major acyl chains including C14, C16:1, and C16, which account for more than 87.5 % of the total fatty alcohol produced at the 72 h time point. At 120 h, the percentage of C14 further increased to about 38 % while C16:1 changed only slightly but C16 dropped to only about 26 % (Fig. 7).

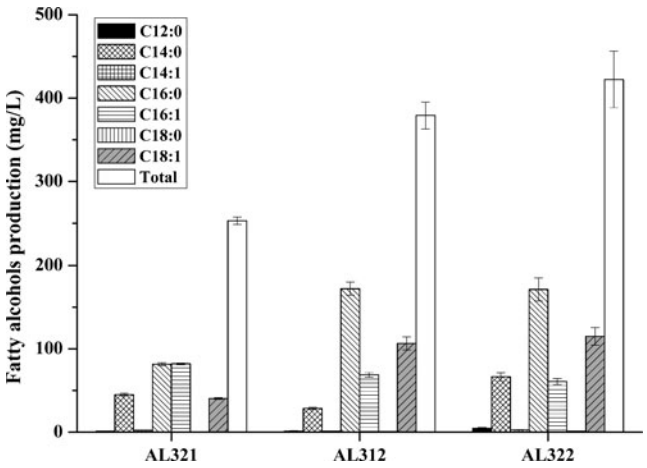

Fig. 6 Fatty alcohol production and carbon chain length distribution of engineered strains AL321 (containing *maqu_2220* in pXT109), AL312 (containing *maqu_2220* in pXT109 and *fadD* in pKC11), and AL322 (containing *maqu_2220* in pXT109, *fadD* in pKC11 and *'tesA*)

Discussion

Fatty alcohol production in engineered *E. coli* has been realized by introducing the fatty acyl-CoA reductase from *A. thaliana* (CER4, At3g11980, At3g44560, At3g56700, and At5g22500; Doan et al. 2009), jojoba (Doan et al. 2009; Zheng et al. 2012), or *A. calcoaceticus* BD413 (Acr1; Steen et al. 2010; Zheng et al. 2012). Especially, 598.6 mg/L fatty alcohols were produced in engineered *E. coli* expressing Acr1 as well as BTE and FadD under fed-batch fermentation condition (Zheng et al. 2012). Although the two recently reported fatty acyl-CoA reductases encoded by *maqu_2507* and *maqu_2220* from *M. aquaeolei* VT8 (Hofvander et al. 2011; Wahlen et al. 2009; Willis et al. 2011) were identified to possess higher catalytic ability for reducing fatty acyl-CoA than Acr1 (Reiser and Somerville 1997), but their ability to heterologously produce fatty alcohols in microbial cells has not been thoroughly examined. Different from these previous reports, Maqu_2507 and Maqu_2220, which could catalyze activated fatty acyl chains directly to the corresponding fatty

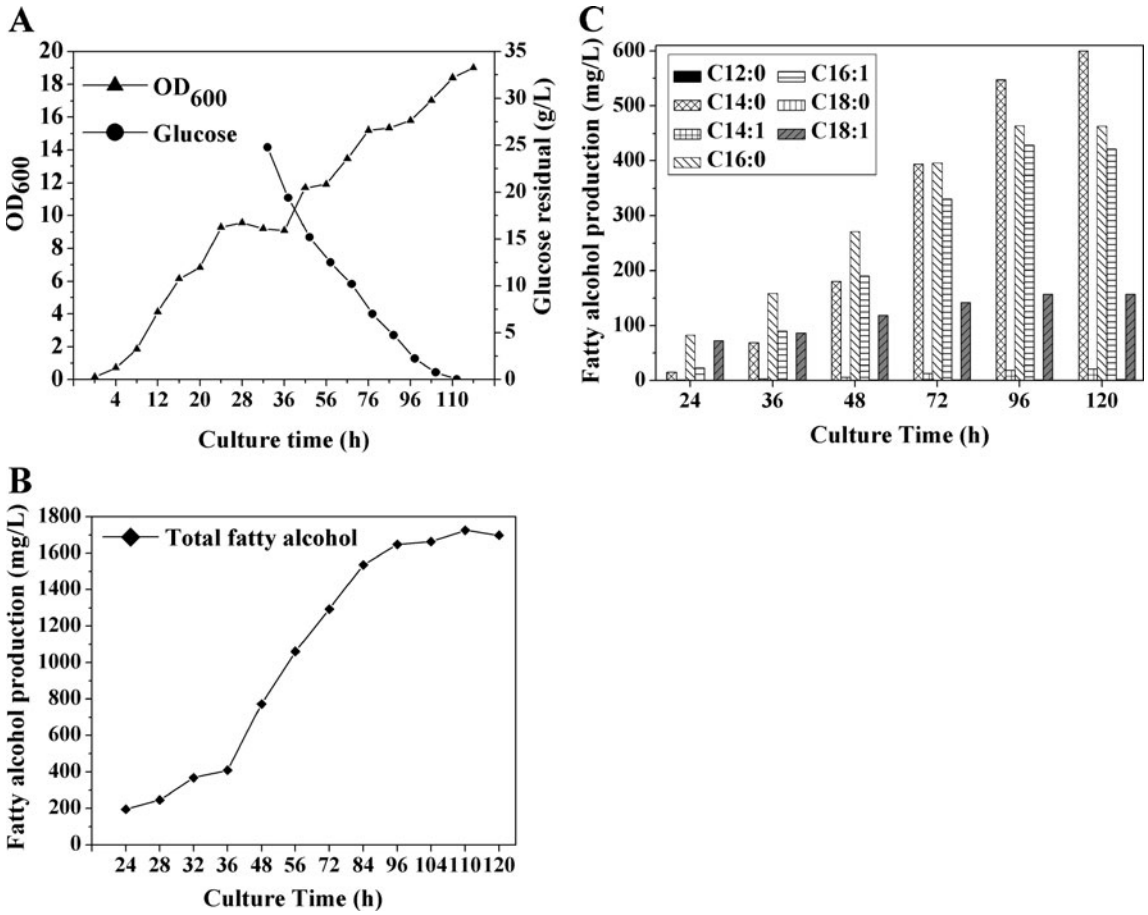

Fig. 7 Time courses of the engineered strain AL322 under batch fermentation: **a** cell growth and glucose consumption, **b** fatty alcohol production, and **c** production of fatty alcohols with different carbon chain length

alcohols without releasing aldehyde intermediate (Hofvander et al. 2011; Wahlen et al. 2009; Willis et al. 2011), were evaluated for fatty alcohol production by analyzing producing capability of *E. coli* expressing each of them with or without feeding of free fatty acids in this study.

In contrast to the Acr1 that only reduce fatty acyl-CoA by two electron to the fatty aldehyde, these two FARs from *Marinobacter* could directly reduce fatty acyl-CoA or acyl-ACP to fatty alcohol without fatty aldehyde intermediate (Hofvander et al. 2011; Reiser and Somerville 1997; Willis et al. 2011). Though FAR from jojoba, which also catalyzing fatty acyl-CoA to fatty alcohol by a four-electron reduction, was overexpressed for fatty alcohol production in *E. coli*, it seems that it has low catalytic activity and carbon-chain specificity incompatible with fatty acyl chain composition of *E. coli* (Metz et al. 2000; Zheng et al. 2012). As the above results of the experiment with the feeding free fatty acids, the both two reductases from *Marinobacter* displayed the wide substrate specificity for C12-C18 fatty acyl chains in *E. coli*. Additionally, Acr1 exhibited higher preference for C12 or C14 fatty acyl-CoA in vivo in this research, which was consistent with the reports by Steen et al. and Zheng et al. but inconsistent with the substrate specificity of an unpurified Acr1 reported by Reiser and Somerville (1997). However, Orf1594 displayed higher chain-length specificity for C16 and C18 fatty acyl chain, which was consistent with the previous study (Schirmer et al. 2010).

The carbon chain length and saturation degree will strongly influence the physicochemical properties of fatty alcohols. Controlling these parameters would potentially allow the synthesis of a range of fatty alcohols with numerous applications. In contrast to the previous work for producing specific C12/C14 and C16/C18 fatty alcohol by two different fatty acyl-CoA reductase (Zheng et al. 2012), fatty alcohols with desired carbon chain length from C12 to C18 and degree of saturation were obtained by co-expressing only one fatty acyl-CoA reductase Maqu_2220 with one or two specific acyl-ACP thioesterases such as TesA, BTE and CCTE in the present study. As previously reported, the results here also show that overexpression of thioesterase can significantly improve fatty acyl-CoA production in *E. coli* and the carbon chain length of fatty alcohol could be tailored by using thioesterases with different substrate specificity combination with Maqu_2220.

As for the titer of total fatty alcohols, 422 mg/L was reached in the *E. coli* mutant strain AL322 by combination of the optimized expression of Maqu_2220 with overexpression of *fadD* and *'tesA* under shake flask condition. To our knowledge the titer of fatty alcohols in AL322 mutant strain we have achieved is higher than the previous reports (Doan et al. 2009; Steen et al. 2010; Zheng et al. 2012), particularly for C16 and C18 fatty alcohols (348.15 mg/L). Steen et al. obtained no more than 60 mg/L C16 and C18 fatty alcohols and 101.5 mg/L C16 and 18 fatty alcohols was obtained by fed-batch fermentation in Zheng's report (Steen et al. 2010; Zheng et al. 2012). Without detailed optimization of fermentation process, an encouraging titer of 1.725 g/L, 2.89-fold of that in Zheng's report (Zheng et al. 2012), was finally achieved by the batch fermentation of the AL322. The yield of 28.3 mg fatty alcohol per gram of glucose is also higher than the maximal yield of 1.0 % obtained by Zheng et al. (2012), but is still far below the theoretical maximal yield and very low for scalable production. Therefore, taking into account for future scalable industrial application, the recombinant strains developed in this study still need to be further improved and optimized for much higher titer of fatty alcohols.

Fatty alcohols usually occur in esterified form (wax esters; Metz et al. 2000; Wahlen et al. 2009). However, fatty alcohols in the free form were also founded in higher organisms (Kanya et al. 2007; Rizzo and Craft 2000). In the case of fatty alcohol-producing cells, the amphipathic nature of fatty alcohols would increase the concerns over their toxicity to the producing cells. Thus, the expression of transporter proteins specific to fatty alcohols might become a feasible solution for improving the cell tolerance to the products as well as releasing the feed-back inhibition of the products. As shown in Fig. S2, there is only 17.1 % fatty alcohol secreting outside the cells of AL322 growing under fermentation condition for 104 h, indicating another way to improve the fatty alcohol production in the future.

In the present study, the changes of fatty alcohol composition during the course of batch fermentation were also examined. As above mentioned, the pattern of fatty alcohol compositions changing with time appears to follow a general trend in that the percentage of C14:0 fatty alcohol increases with cultivation time while the percentage of C16:0 and C18:1 decreases. These results were consistent with previous study on production of free fatty acids by overexpression of acyl-ACP thioesterases from *Ricinus communis* and *Jatropha curcas* in *E. coli* (Li et al. 2012; Zhang et al. 2011). It was speculated that the changes in host cell physiology at different growth phases might lead to variations in the compositions of the precursor pools presented to the acyl-ACP thioesterases (Li et al. 2012; Zhang et al. 2011). When it comes to this study, the variations of fatty alcohol composition may be due to the changes in fatty acid compositions at different growth phases. In summary, given their high activities and broad substrate specificities towards fatty acyl-CoA, these two FARs from *Marinobacter* will be useful catalyst for fatty alcohol production.

Acknowledgments This work was supported by "100-Talent Program of the Chinese Academy of Sciences" foundation (Grant O91001110A).

References

Black PN, DiRusso CC, Metzger AK, Heimert TL (1992) Cloning, sequencing, and expression of the *fadD* gene of *Escherichia coli* encoding acyl coenzyme A synthetase. J Biol Chem 267:25513–25520

Carlsson AS, Yilmaz JL, Green AG, Stymne S, Hofvander P (2011) Replacing fossil oil with fresh oil - with what and for what? Eur J Lipid Sci Technol 113:812–831

Cheng JB (2004) Mammalian wax biosynthesis: I. Identification of two fatty acyl-coenzyme A reductases with different substrate specificities and tissue distributions. J Biol Chem 279:37789–37797

Cho H, Cronan JE Jr (1995) Defective export of a periplasmic enzyme disrupts regulation of fatty acid synthesis. J Biol Chem 270:4216–4219

Davis MS (2000) Overproduction of acetyl-CoA carboxylase activity increases the rate of fatty acid biosynthesis in *Escherichia coli*. J Biol Chem 275:28593–28598

Doan TTP, Carlsson AS, Hamberg M, Bülow L, Stymne S, Olsson P (2009) Functional expression of five *Arabidopsis* fatty acyl-CoA reductase genes in *Escherichia coli*. J Plant Physiol 166:787–796

Domergue F, Vishwanath SJ, Joubes J, Ono J, Lee JA, Bourdon M, Alhattab R, Lowe C, Pascal S, Lessire R, Rowland O (2010) Three *Arabidopsis* fatty acyl-Coenzyme A reductases, FAR1, FAR4, and FAR5, generate primary fatty alcohols associated with suberin deposition. Plant Physiol 153:1539–1554

Duan Y, Zhu Z, Cai K, Tan X, Lu X (2011) De novo biosynthesis of biodiesel by *Escherichia coli* in optimized fed-batch cultivation. PLoS One 6:e20265

Fortman JL, Chhabra S, Mukhopadhyay A, Chou H, Lee TS, Steen E, Keasling JD (2008) Biofuel alternatives to ethanol: pumping the microbial well. Trends Biotechnol 26:375–381

Gervajio GC (2005) Fatty acids and derivatives from coconut oil. Bailey's Industrial Oil and Fat Products. John Wiley & Sons, Inc

Hofvander P, Doan TTP, Hamberg M (2011) A prokaryotic acyl-CoA reductase performing reduction of fatty acyl-CoA to fatty alcohol. FEBS Lett 585:3538–3543

Jiang P, Cronan JE Jr (1994) Inhibition of fatty acid synthesis in *Escherichia coli* in the absence of phospholipid synthesis and release of inhibition by thioesterase action. J Bacteriol 176:2814–2821

Kaczmarzyk D, Fulda M (2010) Fatty acid activation in cyanobacteria mediated by acyl-acyl carrier protein synthetase enables fatty acid recycling. Plant Physiol 152:1598–1610

Kalscheuer R, Stolting T, Steinbuchel A (2006) Microdiesel: *Escherichia coli* engineered for fuel production. Microbiology 152:2529–2536

Kameda K, Nunn WD (1981) Purification and characterization of acyl coenzyme A synthetase from *Escherichia coli*. J Biol Chem 256:5702–5707

Kanya TCS, Rao LJ, Sastry MCS (2007) Characterization of wax esters, free fatty alcohols and free fatty acids of crude wax from sunflower seed oil refineries. Food Chem 101:1552–1557

Li M, Zhang X, Agrawal A, San K-Y (2012) Effect of acetate formation pathway and long chain fatty acid CoA-ligase on the free fatty acid

production in *E. coli* expressing acy-ACP thioesterase from *Ricinus communis*. Metab Eng 14:380–387

Liu T, Vora H, Khosla C (2010) Quantitative analysis and engineering of fatty acid biosynthesis in *E. coli*. Metab Eng 12:378–386

Liu XSJ, Curtiss R (2011) Fatty acid production in genetically modified cyanobacteria. Proc Natl Acad Sci USA 6899–6904

Lu X, Vora H, Khosla C (2008) Overproduction of free fatty acids in *E. coli*: implications for biodiesel production. Metab Eng 10:333–339

Metz JG, Pollard MR, Anderson L, Hayes TR, Lassner MW (2000) Purification of a jojoba embryo fatty acyl-coenzyme A reductase and expression of its cDNA in high erucic acid rapeseed. Plant Physiol 122:635–644

Peralta-Yahya PP, Zhang F, del Cardayre SB, Keasling JD (2012) Microbial engineering for the production of advanced biofuels. Nature 488:320–328

Reiser S, Somerville C (1997) Isolation of mutants of *Acinetobacter calcoaceticus* deficient in wax ester synthesis and complementation of one mutation with a gene encoding a fatty acyl coenzyme A reductase. J Bacteriol 179:2969–2975

Rizzo WB, Craft DA (2000) Sjögren–Larsson syndrome: accumulation of free fatty alcohols in cultured fibroblasts and plasma. J Lipid Res 41:1077–1081

Rowland O, Zheng H, Hepworth SR, Lam P, Jetter R, Kunst L (2006) *CER4* encodes an alcohol-forming fatty acyl-coenzyme A reductase involved in cuticular wax production in Arabidopsis. Plant Physiol 142:866–877

Schirmer A, Rude MA, Li X, Popova E, del Cardayre SB (2010) Microbial biosynthesis of alkanes. Science 329:559–562

Steen EJ, Kang Y, Bokinsky G, Hu Z, Schirmer A, McClure A, del Cardayre SB, Keasling JD (2010) Microbial production of fatty-acid-derived fuels and chemicals from plant biomass. Nature 463:559–562

Tan X, Yao L, Gao Q, Wang W, Qi F, Lu X (2011) Photosynthesis driven conversion of carbon dioxide to fatty alcohols and hydrocarbons in cyanobacteria. Metab Eng 13:169–176

Teerawanichpan P, Qiu X (2010) Fatty acyl-CoA reductase and wax synthase from *Euglena gracilis* in the biosynthesis of medium-chain wax esters. Lipids 45:263–273

Teerawanichpan P, Robertson AJ, Qiu X (2010) A fatty acyl-CoA reductase highly expressed in the head of honey bee (*Apis mellifera*) involves biosynthesis of a wide range of aliphatic fatty alcohols. Insect Biochem Mol Biol 40:641–649

Vioque J, Kolattukudy PE (1997) Resolution and purification of an aldehyde-generating and an alcohol-generating fatty acyl-CoA reductase from pea leaves (Pisum sativum L.). Arch Biochem Biophys 340:64–72

Voelker TA, Davies HM (1994) Alteration of the specificity and regulation of fatty acid synthesis of *Escherichia coli* by expression of a plant medium-chain acyl-acyl carrier protein thioesterase. J Bacteriol 176:7320–7327

Wahlen BD, Oswald WS, Seefeldt LC, Barney BM (2009) Purification, characterization, and potential bacterial wax production role of an NADPH-dependent fatty aldehyde reductase from *Marinobacter aquaeolei* VT8. Appl Environ Microbiol 75:2758–2764

Willis RM, Wahlen BD, Seefeldt LC, Barney BM (2011) Characterization of a fatty acyl-CoA reductase from *Marinobacter aquaeolei* VT8: a bacterial enzyme catalyzing the reduction of fatty acyl-CoA to fatty alcohol. Biochemistry 50:10550–10558

Yuan L, Voelker TA, Hawkins DJ (1995) Modification of the substrate specificity of an acyl-acyl carrier protein thioesterase by protein engineering. Proc Natl Acad Sci USA 92:10639–10643

Zhang F, Ouellet M, Batth TS, Adams PD, Petzold CJ, Mukhopadhyay A, Keasling JD (2012) Enhancing fatty acid production by the expression of the regulatory transcription factor FadR. Metab Eng 14:653–660

Zhang X, Li M, Agrawal A, San K-Y (2011) Efficient free fatty acid production in *Escherichia coli* using plant acyl-ACP thioesterases. Metab Eng 13:713–722

Zheng YN, Li LL, Liu Q, Yang JM, Wang XW, Liu W, Xu X, Liu H, Zhao G, Xian M (2012) Optimization of fatty alcohol biosynthesis pathway for selectively enhanced production of C12/14 and C16/18 fatty alcohols in engineered *Escherichia coli*. Microb Cell Fact 11:65