

Boosting fatty acid synthesis in *Rhodococcus opacus* PD630 by overexpression of autologous thioesterases

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Abstract

Objectives To explore the role of thioesterases in *Rhodococcus opacus* PD630 by endogenously overexpression in this bacteria for increased lipid production.

Results Overexpression of four thioesterases from *R. opacus* PD630 in *E. coli* led to a 2- to 8-fold increase in C16:1 and C18:1 fatty acids while, when overexpressed in *R. opacus* PD630, only two recombinants had significant effect on the quantities and compositions of total fatty acid. The contents of total fatty acids (FAs) in two recombinants, pJTE2 (OPAG_00508 thioesterase) and pJTE4 (WP_012687673.1 thioesterase), were 400–460 mg/g (CDW) which is 1.5 times of

wild-type strain PD630 (300–350 mg/g CDW), and 20–30 % (w/w) more than that of the control strain PDpJAM2 (330–370 mg/g CDW). The contents of 17:1 and 18:1 fatty acids increased by about 27 and 35 %, respectively, in pJTE2 and by 35 and 20 %, respectively, in pJTE4 compared with the control strain.

Conclusions The engineered strains showed improved production of lipid (as total fatty acids), and could also tailor the composition of the fatty acid profile when cultured in mineral salts medium using glucose as sole carbon source.

Keywords Acyl-ACP thioesterase · *E. coli* · Lipid production · *Rhodococcus opacus* PD630 · Substrate specificity · Total fatty acid

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Introduction

Microbial lipids are increasingly being considered as sources for biodiesel or high-valued fatty acids (FAs). Major efforts have been focused on microbial production of lipids by using metabolic and genetic engineering tools. *Rhodococcus opacus* PD630, as a model oleaginous prokaryote, can accumulate large amount of neutral lipids up to 25 % (w/w) of its cell dry weight when cultivated in the mineral salt medium using glucose as the sole carbon source (Alvarez et al. 2008). Studies on lipid metabolism in *R. opacus* PD630 have focused on the synthesis of triacylglycerols (TAG). For

example, *R. opacus* PD630 overexpressing diacylglycerol acyltransferase (DGAT) or phosphatidic acid phosphatase (PAP) had an increase of 10–15 % (w/w) in TAG content (Hernandez et al. 2013, 2015). However, the regulation of genes involved in fatty acid (FA) biosynthesis in PD630 has not been reported.

Thioesterases (TE) hydrolyze the thioester bond between a carbonyl group and a sulfur atom of acyl-ACP and play an essential role in FA biosynthesis. Based on their substrate specificities, TEs are classified into EC 3.1.2.1 to EC 3.1.2.27 as well as EC 3.1.2.- for unclassified TEs. EC 3.1.2.14 enzymes occur in both eukaryotes and bacteria and show fatty acyl–acyl carrier protein (ACP) hydrolase activity. In bacteria, the FA biosynthesis pathway occurs in the cytoplasm and is composed of a repeated cycle of reactions involving the sequential condensation of two-carbon units onto a growing fatty acyl-ACP derivatives. The successive elongation of the acyl-ACP chain can be terminated by acyl-ACP TEs (E.C. 3.1.2.14) that hydrolyze the thioester bond of acyl-ACP to release the FAs. The FAs are then converted to fatty acyl-CoAs that are further converted to TAGs and other lipids for storage within the cell.

The production and composition of FAs are determined by acyl-ACP TEs. Many acyl-ACP TEs from plants have been heterologously-expressed in *Escherichia coli*. For example, acyl-ACP TE genes from *Cuphea* were overexpressed in *E. coli* to produce high content of specific chain length FAs (Joseph et al. 2013). Expression of acyl-ACP TE cDNA from *Umbellularia californica* in *E. coli* resulted in a very high accumulation of 12:0 which is a minor FA in the wild type strain (Fan et al. 2013). In addition, the overexpression of two plant TEs from *Ricinus communis* and *Jatropha curcas* in *E. coli* increased total FAs content from 0.2 g/l to 2 g/l (Zhang 2011). Although acyl-ACP TEs from plants have been extensively reported, little attention has been paid to bacterial TEs.

Like plant acyl-ACP TEs, bacterial acyl-ACP TEs can also increase the content of total FA and have broad substrate specificities for FAs with various chain lengths (Lee 2013). A codon-optimized acyl-ACP TE of *Streptococcus pyogenes* overexpressed in *E. coli* yielded 339 mg total FA/l, which was 1.5-fold higher than that of the control strain (Lee 2013). Overexpression of *Acinetobacter baylyi* TE (AcTesA) achieved 500 mg FA/l in shake-flask culture, in

contrast to only 20.5 mg/l obtained in wild-type *E. coli* (Zheng 2012). Bacterial acyl-ACP TE may provide superior FA production compared to plant acyl-ACP TE, given that bacterial proteins can be more optimally expressed in bacterial cell than plant proteins (Jing et al. 2011). Thus, in this study, the genes of one putative acyl-ACP TE (OPAG_00508) and three E.C 3.1.2.14 enzymes (EHI47208.1, WP_005241865.1 and WP_012687673.1) from *R. opacus* PD630, which have not been studied before, were chosen and homologously overexpressed in PD630. The substrate-specificity of these proteins and their influence on FAs synthesis in *R. opacus* PD630 were studied and are reported here.

Materials and methods

Strains and medium

The sources of all bacteria are listed in Table 1. *E. coli* Top10 was used for plasmid copy and storage. *Rhodococcus opacus* PD630 and *E. coli* BL21(DE3) transformed with recombinant plasmids were used as host strain for protein expression and the production of lipids. Lysogeny broth (LB) was used as seed medium to culture *R. opacus* strains and *E. coli* strains. For the production of FAs, *R. opacus* strains were cultivated in the mineral salts medium (MSM) as described by Schlegel et al. (1961). Glucose was used in MSM as the sole carbon source at 1 % (w/v). For nitrogen-limiting conditions, NH₄Cl was decreased to 0.1 g/l (MSM0.1/1.0). *E. coli* strains were cultivated in M9 to produce FAs. SOC medium (20 g tryptone/l, 5 g yeast extract/l, 0.5 g NaCl/l, 2.5 mM KCl, 10 mM MgCl₂, 20 mM glucose) was used for recovery of competent cells after electroporation.

Plasmid construction and transformation

Plasmids and primers used in this study are listed in Supplementary Table 1. All DNA manipulations were performed according to standard procedures. Chromosomal DNA of *R. opacus* PD630 was isolated and used as template for PCR. The DNA fragments of four putative TEs from *R. opacus* PD630 were amplified without its native start codon and stop codon using specific primers (Supplementary Table 1), and inserted into *Bam*HI/*Xba*I sites of pJAM2 to generate

Table 1 List of strains used

Strains	Description	Reference/source
<i>E. coli</i> Top10	F- <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA1 araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Str ^R) <i>endA1 nupG</i>	Invitrogen
<i>R. opacus</i> PD630	Parental strain	DSM 44193
PDPJAM2	PD630 derivative carrying pJAM2 plasmid, used as control strain; Km ^R	This study
PJTE1	PD630 derivative carrying pJAM2/TE1 plasmid; Km ^R	This study
PJTE2	PD630 derivative carrying pJAM2/TE2 plasmid; Km ^R	This study
PJTE3	PD630 derivative carrying pJAM2/TE3 plasmid; Km ^R	This study
PJTE4	PD630 derivative carrying pJAM2/TE4 plasmid; Km ^R	This study
BL21(DE3)	F ⁻ <i>ompT gal dcm lon hsdSB</i> (<i>r_B m_B</i>) λ (DE3)	Invitrogen
BL1	BL21(DE3) derivative carrying pCold1 plasmid; Amp ^R	This study
BLTE1	BL21(DE3) derivative carrying pCold1/TE1 plasmid; Amp ^R	This study
BLTE2	BL21(DE3) derivative carrying pCold1/TE2 plasmid; Amp ^R	This study
BLTE3	BL21(DE3) derivative carrying pCold1/TE3 plasmid; Amp ^R	This study
BLTE4	BL21(DE3) derivative carrying pCold1/TE4 plasmid; Amp ^R	This study

pJAM2/TEs (pJAM2/TE1, pJAM2/TE2, pJAM2/TE3 and pJAM2/TE4) which contains an inducible acetamidase (*ace*) promoter and six histidine codons downstream of the *Xba*I site (Triccas et al. 1998). The TE gene in the constructed fusion lacked its own stop codon but contained the stop codon after the His6-tag linker sequence of pJAM2. Therefore, the amino acids SRHHHHHH occurred in the C-terminal region of the protein (Hanisch et al. 2006). To obtain pCold1/TEs, the DNA fragments of four putative TEs were inserted into *Bam*HI/*Hind*III sites of pCold1.

Plasmid pJAM2 and its derivatives were introduced into *R. opacus* via electroporation (Shao et al. 1995; Kalscheuer et al. 1999) with minor modifications. To obtain electrocompetent cells of *R. opacus*, 2 ml LB pre-culture grown at 28 °C for 24 h were inoculated into 50 ml LB supplemented with 8.5 g glycine/l and 10 g sucrose/l in a 250 ml flask. Before electroporation, 200 μ l competent cells were mixed with DNA and pre-incubated at 40 °C for 5 min followed by holding for 10 min on ice and then electroporated. Electroporation was performed in an electrocuvette (Bio-Rad) with gaps of 2 mm and the following settings: 2.5 kV/cm, 600 Ω and 25 μ F. Time constants of 6 ms were used. Pulsed cells were immediately diluted with 800 μ l SOC medium and regenerated at 28 °C for 3–4 h before they were plated on solid SOC agar plate containing kanamycin (50 μ g/ml).

Transformants could be scored after 3–4 days. Plasmid pCold1 and its derivatives were introduced into *E. coli* BL21 (DE3) by heat shock at 42 °C for 90 s. Transformants were grown overnight on LB agar plates containing ampicillin.

Cultivation conditions in shake-flasks

For preparation of the inoculum, *R. opacus* PD630 recombinant strains were cultured aerobically in liquid LB containing kanamycin (50 μ g/ml) for 24 h at 28 °C with shaking at 200 rpm. These cultures were inoculated at 1 % (v/v) into 1 l shake-flasks with liquid MSM0.1/1.0 containing corresponding antibiotics and an appropriate concentration of acetamide (0.5 % w/v) for the induction of the acetamidase (*ace*) promoter of pJAM2 and its derivatives (Triccas et al. 1998). Cells were harvested and washed twice with sterile saline (0.85 % w/v NaCl) before analysis. *E. coli* BL21(DE3) recombinant strains were grown in LB medium containing ampicillin (100 μ g/ml) for 12 h at 37 °C with shaking at 200 rpm. This was used at 1 % (v/v) to inoculate 1 l shake-flasks with liquid M9 medium. When the OD₆₀₀ reached 0.5–0.6, IPTG was added at 0.1 mM to induce the expression of the pCold1. Cells were grown for additional 24 h at 16 °C and were harvested by centrifugation.

Preparation of crude cell extracts and soluble fractions, SDS-PAGE, and western blot analysis

Cells of recombinant strains were harvested by centrifugation ($4000\times g$) for 15 min at 4 °C, washed twice with sterile saline (0.85 % w/v NaCl), and suspended in 500 μ l 50 mM sodium phosphate buffer (pH 7.4). All procedures were carried out in ice to avoid changes in lipids. Subsequently, cells were lysed by ultrasonication on the ice. In order to obtain the whole cell extracts, the lysates obtained were centrifuged as described above to remove residual cells. The cytoplasmic components were obtained by centrifuging the whole cell extracts at $100,000\times g$ at 4 °C for 30 min. Protein concentrations were determined by the Bradford method using bovine serum albumin as standard. All crude cell extracts and soluble fractions were added with loading buffer. Proteins were denatured by holding at 100 °C for 5 min and separating using 12 % (w/v) SDS-PAGE. Proteins were stained with Coomassie Brilliant Blue R-250. All western blotting was performed as described by Burnette (1981). LifeTein anti-His monoclonal antibodies (LifeTein, Chian) were used as the primary antibody at a dilution of 1:2000–4000. Goat anti-mouse horseradish peroxidase (HRP)-affinipure antibodies (LifeTein, Chian) were used as secondary antibody at a dilution of 1:10,000.

Fatty acid (FA) extraction and analytical techniques

Total FAs were extracted as described previously (Sakuradani et al. 2003): lyophilized cells were mixed with 2 ml 6 M HCl and held at 80 °C for 3 h. Tridecanoic acid (13:0) was added into the acidified samples as internal standard. Three ml chloroform/methanol (2:1, v/v) was added to extract lipid. The mixture was vortexed for 15 min and then centrifuged at $3000\times g$ for 5 min. The chloroform phase was evaporated under N_2 . Samples were methylated using 1 ml 10 % (w/w) HCl/methanol at 60 °C for 3 h. The fatty acid methyl esters (FAMES) were quantified by GC equipped with a flame ionization detector (FID). The separation of FAMES was performed using a PB-wax column (30 m \times 0.32 mm; 0.25 μ m film thickness). The oven was initially held at 120 °C for 3 min, and raised with a gradient of 5 °C/min to 190 °C, then raised with a gradient of 4 °C/min to 220 °C for

2 min. N_2 was used as the carrier gas. The injector and detector were held at 240 °C and 260 °C, respectively.

Results and discussion

Selection of four TEs

Based on the classification of the thioesterases from *R. opacus* PD630, as given on ThYme database (<http://www.enzyme.cbirc.iastate.edu>), the three thioesterases—EHI47208.1 (TE1), WP_005241865.1 (TE3) and WP_012687673.1 (TE4) were selected, and they were all belong to EC 3.1.2.14 family. Bacterial thioesterases in this family were acyl-ACP thioesterase (Cantu 2010). The one putative acyl-ACP TE OPAG_00508 (TE2) was not included in ThYme database, but selected through a multiple amino acid sequence alignment, which was built by aligning the sequences of OPAG_00508 and 12 publically-available acyl-ACP TEs from other *Rhodococcus* species. The OPAG_00508 amino acid sequence shared similarity with its orthologs from *Rhodococcus* sp. JVH1 (98 % identity), *Rhodococcus* sp. AD45 (64 %), *R. erythropolis* SK121 (65 %), *R. equi* ATCC 33707 (56 %) and *R. rhodochrous* ATCC 21198 (53 %). In addition, we identified the conserved domain of these TEs—see Fig. 1. This result demonstrated that the OPAG_00508 from *R. opacus* PD630 probably belongs to acyl-ACP TE group.

Expression of the four TEs in *E. coli* BL21(DE3)

Escherichia coli BL21(DE3) was used for the expression of four TEs (TE1, TE2, TE3 and TE4) from *R. opacus* PD630 to identify enzyme activities. To obtain functionally active TEs, we used pCold1 as expression vector that can express proteins at low temperatures to improve the solubility of TEs in *E. coli* BL21(DE3). The recombinant *E. coli* BL21(DE3) strains were cultured at 16 °C for 24 h in induced medium, with the OD_{600} of all strains reaching approx 5 to 6 to make sure the consistency of growth status.

Total FA composition analysis (Fig. 2b) showed that the recombinant *E. coli* BL21(DE3) strains expressing TE1, TE2, TE3 and TE4 alone had considerable increases in contents of 16:1 and 18:1 by 2- to 3-fold and 4- to 8-fold, respectively, and led to a 25–50 % decrease in the content of 16:0 as compared to strain BL1. The results demonstrated that the four

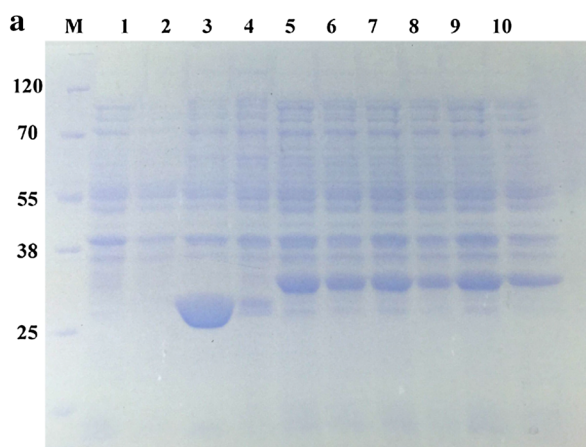
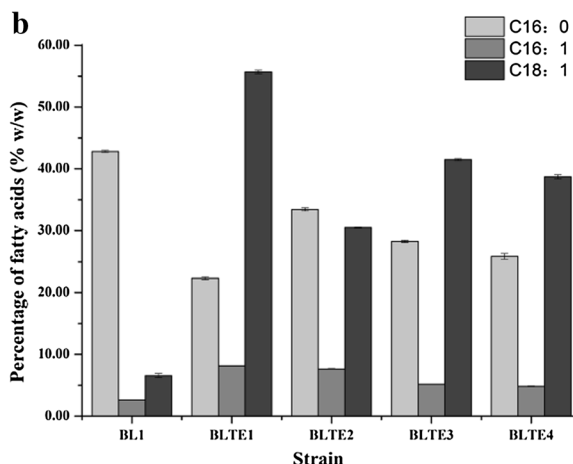


Fig. 2 a SDS-PAGE analysis of crude cell extracts and soluble fractions from the recombinant *E. coli* strains. Lane M protein molecular weight marker; lanes 1, 3, 5, 7, 9 whole cell proteins



of BL1, BLTE1, BLTE2, BLTE3 and BLTE4; lanes 2, 4, 6, 8, 10: soluble fractions of recombinants. **b** The relative proportion of fatty acids (16:0, 16:1, 18:1) in recombinants

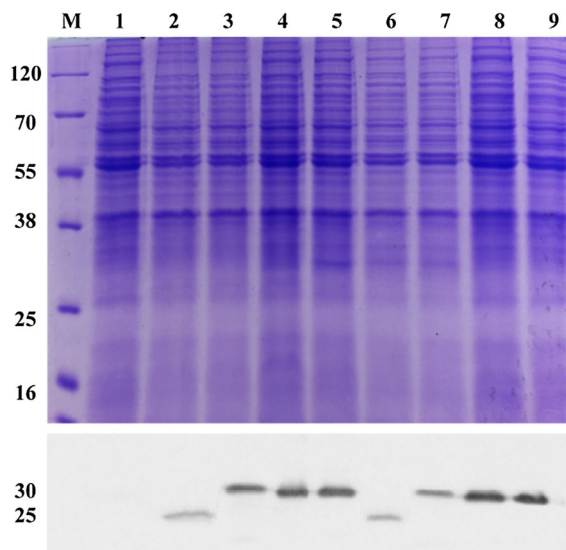


Fig. 3 SDS-PAGE and western blot analysis of crude cell extracts and soluble fractions from the recombinant *R. opacus* PD630 strains. Lane M, protein molecular weight marker; lane 1 whole cell extracts of the control strain; lanes 2–5 whole cell proteins of pJTE1, pJTE2, pJTE3 and pJTE4, respectively; lanes 6–9 cytosol components of four recombinants

opacus than in *M. smegmatis*. However, the results of western blot analysis showed a single protein band on each track from the recombinant strains except for PDpJAM2 (Fig. 3). The EHI47208.1 protein molecular mass was the smallest one at ~25 kDa; OPAG_00508 was at ~30 kDa; the other two proteins molecular mass were similar at ~29 kDa.

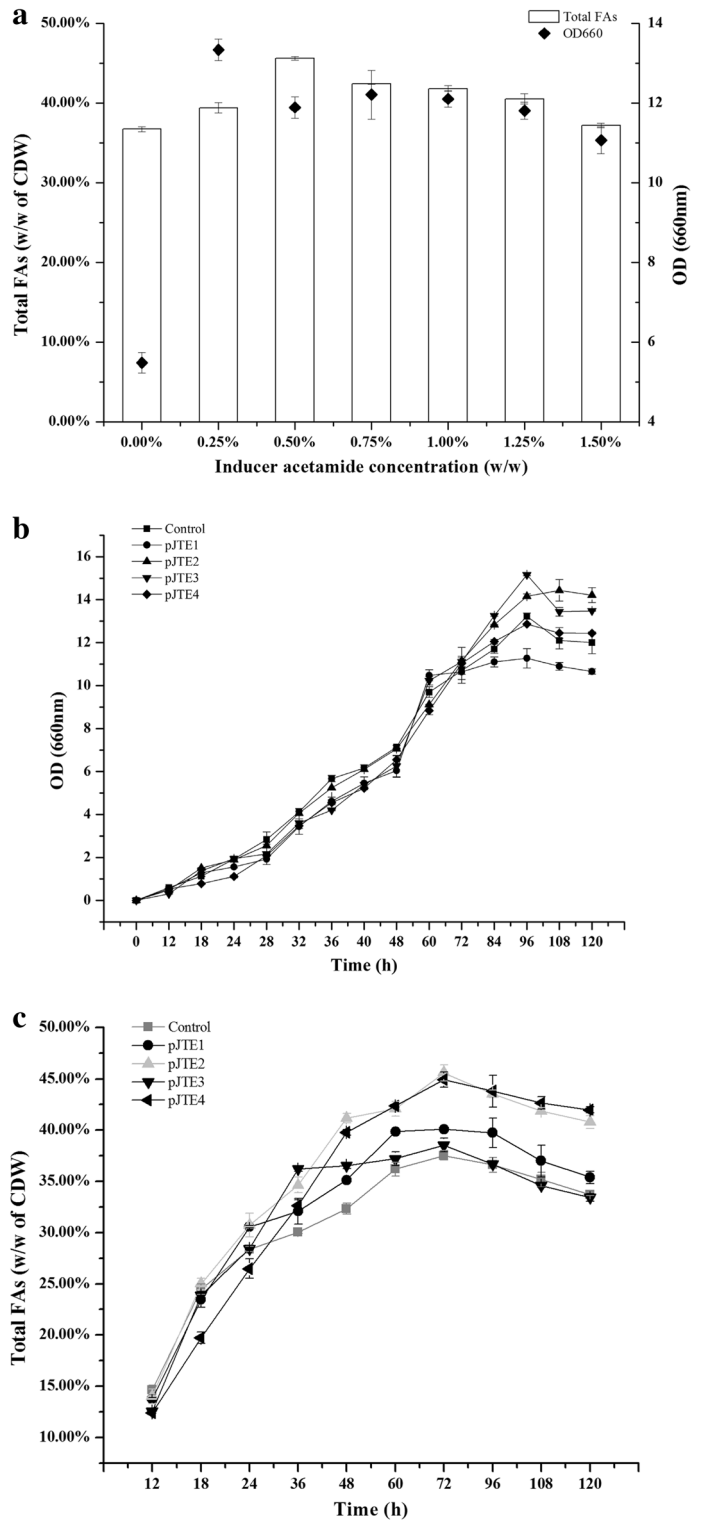
Thus the TEs were indeed expressed as soluble intracellular proteins. The soluble products of the expressed proteins suggest that they are in an active form. This may be partly due to their low expression in the recombinant strains and also that bacterial proteins can be more optimally expressed in their own cells than in an exogenous host. Furthermore, the synthesis of FAs also occurs in the cytoplasm where the TEs can play an effective role.

Effects of acetamide concentration on cell growth and total FA accumulation

Acetamide as inducer was added into the medium to promote the expression of pJAM2 plasmid. To study the effects of acetamide concentration on the total FA accumulation, strain pJTE2 was cultured in 50 ml MSM0.1/1.0 containing acetamide from 0.25 to 1.5 % (w/v) and incubated at 28 °C, with shaking at 200 rpm for 72 h. The optimum concentration of acetamide was 0.5 % (w/v) and the content of total FA with this concentration was 45.6 % (w/w) of CDW which was 24 % higher than that in uninduced culture (Fig. 4a). The increased content of total FAs in pJTE2 cells grown with acetamide is more likely to be associated with a more active acyl-ACP TE.

Oleaginous microorganisms cultivated in the nitrogen-limited medium accumulate more lipids than in nitrogen-rich medium, when their growth is restricted (Ratledge and Wynn 2002). The maximum cell dry

Fig. 4 a The content of total fatty acid produced by recombinant pJTE2 and the corresponding OD₆₆₀ values ($y = 0.5122 \times g/l$, y : cell dry weight, x : optical density) with various concentrations of acetamide. **b** Growth and **c** total fatty acids contents of four recombinants and the control strain. Values were mean of two independent experiments. *Error bars* represent the standard error of the mean



weight of pJTE2 reached to 2.56 g/l when growing in MSM0.1/1.0 without acetamide, which was similar to wild-type strain PD630. However, when growing the bacteria in MSM0.1/1.0 with addition of 0.25 or 0.5 % acetamide, the cell dry weight reached to 6.81 and 6.15 g/l respectively. This result demonstrated that the addition of acetamide has a positive influence on the growth of the strains possibly by increasing the amount of available N to the cells.

Effect of TEs overexpression on the cell growth and FA accumulation in *R. opacus* PD630

To determine whether the overexpression of the four TEs could boost the total FA synthesis and affect the growth status of cells, we determined the total FA yield and composition as well as the cell dry weight of the control strain and recombinant strains. The engineered *R. opacus* PD630 strains were cultivated in MSM0.1/1.0 and induced with acetamide (0.5 %, w/v). Figure 4b shows that the growth rate of PDpJAM2 was similar to that of pJTE1, pJTE2, pJTE3 and pJTE4 until the late growth phase (~72 h). This was expected as cells would not have had to divert too many resources to produce the low-expressed TEs. However, after the main growth phase, the growth rate and the final OD₆₆₀ of strains pJTE2 and pJTE3 were a little higher than that of PDpJAM2. These results suggest that the overexpression of each of the four TEs did not have an inhibitory effect on the growth of engineered strains.

TEs have substrate specificities and can hydrolyze the thioester bond of oleoyl-ACP, which then regulates the FA composition of strain. Thus, the intracellular activity of TE to some degree can be confirmed by FA analysis. As shown in Fig. 5, decreases in amounts of 16:0 in pJTE2 and pJTE4 strains were more significant than that of PDpJAM2 over time and, at the same time, the contents of 17:1 and 18:1 increased by 27 and 35 %, respectively, in pJTE2, by 35 and 20 %, respectively, in pJTE4 during stationary phase. These changes were greater than seen with PDpJAM2 and wild-type *R. opacus* PD630 (Holder et al. 2011; Alvarez et al. 2000). This is because cells start to accumulate lipids in the stationary phase and TEs then become more effective as there is now more substrate available in cells. However, overexpression of TE2 and TE4 alone did not lead to significant change of FA composition in PD630 compared to that in *E. coli*. Thus, we speculate that, besides the four TEs, other genes or factors might also

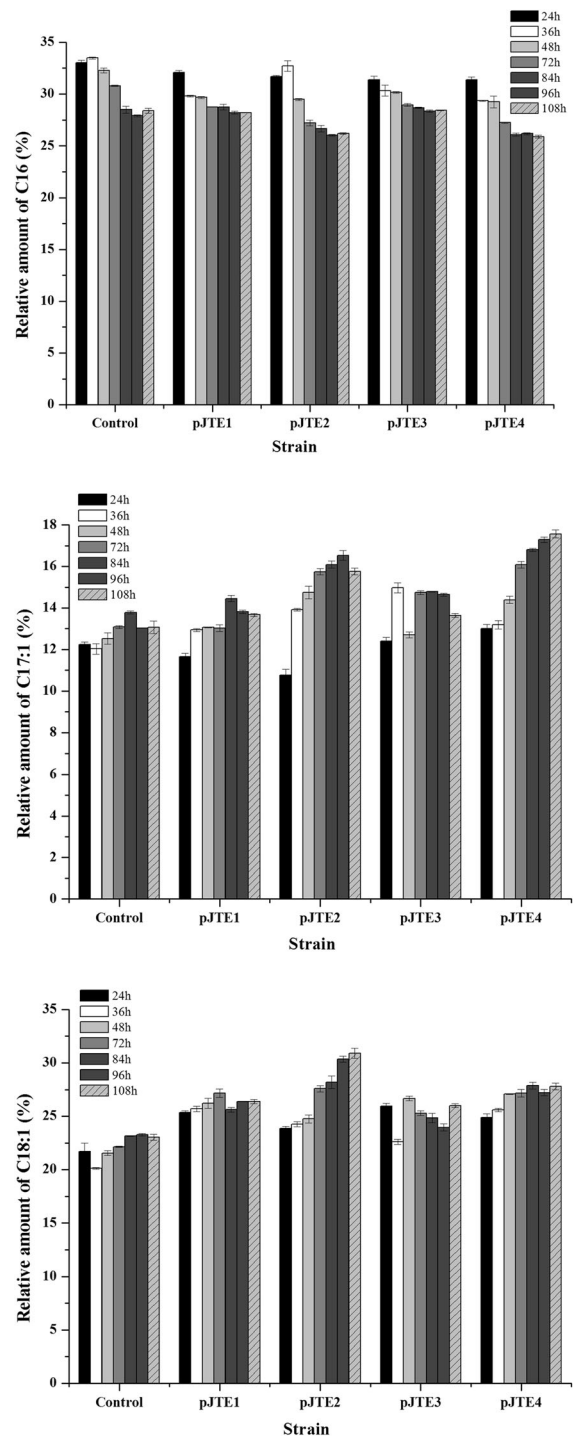


Fig. 5 Contents of 16:0, 17:1 and 18:1 in overexpressing strains of pJTE1, pJTE2, pJTE3 pJTE4 and the control strain PDpJAM2

Table 2 The total fatty acid content and OD₆₆₀ of *R. opacus* PD630 wild-type strain cultivated in MSM medium

Culture conditions	% Total fatty acid content (CDW)	OD ₆₆₀
0 % acetamide 48 h	30.2 ± 0.89	3.7 ± 0.42
0 % acetamide 72 h	35.3 ± 0.63	5.4 ± 0.35
0 % acetamide 96 h	34.6 ± 1.12	6.1 ± 0.50
0 % acetamide 120 h	33.4 ± 0.84	5.8 ± 0.27
0.5 % acetamide 48 h	24.4 ± 1.33	9.3 ± 0.18
0.5 % acetamide 72 h	27.6 ± 0.65	11.8 ± 0.35
0.5 % acetamide 96 h	30.2 ± 0.72	14.5 ± 0.57
0.5 % acetamide 120 h	28.6 ± 0.58	13.2 ± 0.58

be involved in controlling the FA composition in PD630. Overall, the changes in FA composition suggested that TE2 has a preference for 18:1 and that TE4 is more active with 17:1.

The lipid content, expressed as total FAs, of engineered strains pJTE2 and pJTE4 reached, respectively, 41 and 38 % (w/w) of CDW at 48 h, and 46 and 44 % (w/w) of CDW, respectively, at 72 h. As shown in Fig. 4c, quantitative analyses revealed an increase of the lipids, as total FAs, in the recombinant strains pJTE2 and pJTE4 by 15–30 % and 10–25 %, respectively, in comparison to PDpJAM2 after 48 h of cultivation, while pJTE1 and pJTE3 showed no obvious changes.

In order to decouple the impact of the acetamide addition, the wild type strain was cultivated in the absence and presence of 0.5 % acetamide. We found that the wild type strain grown in the absence of acetamide can accumulate more lipids than that in the presence of acetamide, while the latter led to 2–3 times as much biomass (measured as the OD₆₆₀ value) as the former (Table 2). It is suggested that the wild type strain probably uses acetamide for growth and thus the cells cannot accumulate as much lipid in this nitrogen-rich medium. In the presence of acetamide, the pJTE2 and pJTE4 had 1.5 times as much total FAs as the wild type strain. However, the PDpJAM2 also accumulated more lipids than the wild type strain. Thus, we speculate that the PDpJAM2 needs to use some acetamide for inducing the expression of the vector, and the addition of acetamide decreased lipid accumulation in wild type strain more than that in PDpJAM2.

In PD630 cells, FAs are mainly produced through FA synthase system I, and they are probably key

intermediates for the biosynthesis of many of the lipids such as TAGs, phospholipids and wax esters (Holder et al. 2011). For example, the fatty acyl-CoA products originating from FAs are attached to glycerol thereby producing the lipids in PD630 (Holder et al. 2011), and a fatty acid transport protein in *Rhodococcus* strain RHA1 was involved in the provision of FAs to TAG metabolism (Villalba and Alvarez 2014). In addition, the accumulated acyl-ACPs would inhibit upstream enzymes in FA synthesis (Jiang 1994). Therefore, we can utilize the critical function of acyl-TEs in the alleviation of feedback inhibition of acyl-ACPs, during the FA synthesis process, to improve the pool of FAs, which may contribute to the accumulation of lipids in PD630.

Conclusion

The four TEs from *R. opacus* PD630 when heterologously overexpressed in *E. coli* BL21(DE3) led to considerable changes in FA composition. When the four TEs were homologously overexpressed in *R. opacus* PD630, the putative acyl-ACP TE2 (OPAG_00508) and TE4 (WP_012687673.1) led to significant increases in lipid accumulation and evident changes in FA composition. Thus, the two putative acyl-ACP TEs contribute to the production of total FAs and also have a specific influence on the FA composition in *R. opacus* PD630. However, the TE1 (EHI47208.1) and TE3 (WP_005241865.1) did not lead to obvious changes in lipid content and FAs composition.

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Supporting Information Supplementary Table 1—List of plasmids and primers used.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical statement This article does not contain any studies with human participants or animals performed by any of the authors

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