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Cloning and expression of fatty acids biosynthesis key enzymes from sunflower (*Helianthus annuus* L.) in *Escherichia coli*

María Josefa Serrano-Vega, Mónica Venegas-Calerón, Rafael Garcés, Enrique Martínez-Force*

Instituto de la Grasa, CSIC, Av. Padre García Tejero-4, 41012 Sevilla, Spain

Abstract

To further characterize the stearoyl-acyl carrier protein (ACP) desaturase (EC 1.14.99.6) and the acyl-ACP thioesterase FatB (EC 3.1.2.14) activities from sunflower seeds, we cloned, sequenced and expressed the recombinant genes in *Escherichia coli*. We obtained two partially purified proteins, His-SAD and His-FATB, each of about 45 000 Da. The expression of either proteins produced changes in the *E. coli* fatty acid profile indicating the functionality of the recombinant proteins. While the expression of His-SAD produced an effect similar to that produced by overexpression of the *fab*A gene, responsible for the fatty acid desaturation in *E. coli*, the expression of His-FATB gave rise to an unbalance between unsaturated fatty acids and a toxic effect in *E. coli*.

Keywords: Cloning; Expression, *Escherichia coli*; Purification; Fatty acids; Stearoyl-acyl carrier protein desaturase

1. Introduction

Vegetable oils mostly contain unsaturated fatty acids in their lipids, but for specific food purposes, a higher content of more-saturated fatty acids is needed. Mutant lines with increased levels of saturated fatty acids, palmitic and stearic, have been obtained in *Arabidopsis thaliana* [1], *Glycine max* [2] and *Helianthus annuus* [3–5]. Besides their food-technological applications, these mutants are very useful for studying the biochemical pathway of fatty acid biosynthesis.

The pathway of fatty acid synthesis in oilseeds starts with malonyl-CoA, the product of acetyl-CoA carboxylase, and by means of repeated condensations of malonyl-acyl carrier protein (ACP), mediated by the fatty acid synthetases III and I (FAS III and FAS I) [6,7], short and medium chain (C8–C14) fatty acids as well as palmitic acid are synthesised. Palmitoyl-ACP can be exported out of the plastid or elongated to stearoyl-ACP by the enzymatic complex fatty acid synthetase II (FAS II) [8]. Stearoyl-ACP can be exported out of the plastid or desaturated to oleic acid by stearoyl-ACP desaturase (SAD) [9]. In oilseeds, oleyl-ACP is the major final product of ''de novo'' fatty acid biosynthesis in the plastids, accounting for 80–90% of the total. Export of acyl-ACPs out of the plastid requires the hydrolysis mediated by acyl-ACP thioesterases, which separate the fatty acid from the ACP. This step seems to limit the rate of export of fatty acids.

The stearoyl-ACP Δ^9 desaturase is a soluble homodimer that introduces the oleic acid double

*Corresponding author.
E-mail address: emforce@cica.es (E. Martínez-Force).

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bond in the presence of O_2, NAD(P)H, NAD(P)H ferredoxin oxidoreductase and ferredoxin [10]. A combination of spectroscopic [11] and crystallographic studies [12] has established that each mature subunit of SAD is a single 42 000 polypeptide. Comparison of the amino acid sequences deduced from cDNA clones [13,14] revealed that SAD from distantly related plant species is a highly conserved polypeptide. In contrast, no substantial homology was observed with the corresponding enzymes from animals [15]. Several acyl-ACP desaturase genes have been cloned and expressed in *E. coli* in order to characterize their products [14,16,17].

Acyl-ACP thioesterases terminate acyl-chain elongation during fatty acid biosynthesis hydrolysing the thioester bond of acyl-ACP [18]. The substrate specificity of acyl-ACP thioesterases play a major role in determining when the acyl chains are cleaved and released from the ACPs, and thus, directly influence the pools of free fatty acids available for export to extraplastidic compartments. The plant acyl-ACP thioesterases are soluble homodimers of polypeptides of between 360 and 390 amino acid residues, with a molecular mass ranging from 34 000 to 40 000. Acyl-ACP thioesterases are usually categorized into two groups depending on sequence identity and, in some cases, acyl-ACP preference. Jones et al. [19] originally classified the two different types of thioesterases as FatA or FatB, with substrate preferences for unsaturated acyl-ACPs or saturated acyl-ACP, respectively. Some thioesterase genes have been cloned and expressed in *E. coli* in order to biochemically characterize their products [19–23]. Expression in *E. coli* of a FatB thioesterase from California bay tree, results in the accumulation of a massive amount of lauric acid in the culture medium [20].

Whereas the major product of the intraplastidial ''de novo'' fatty acids biosynthesis is oleic acid and to a lesser extent palmitic and stearic acids in wild type sunflower, high saturated lines present higher amounts (up to 30%) of palmitoyl-ACP or stearoyl-ACP [24]. Enzymatic data from fifteen days after flowering (DAF) sunflower seeds showed an alteration of stearoyl-ACP desaturase and thioesterase activities in high stearic mutants [25]. Additionally, experimental data indicate the existence of a dynamic channelling of substrates during the final steps of ''de novo'' fatty acids biosynthesis [26]. This model assumes the interaction between enzymatic FAS I/FAS II and FAS II/SAD complexes respectively, thus implying that there must be a region on SAD involved in the temporal interaction with FAS II. This idea is corroborated by recent experiments that, using the yeast two-hybrid system, suggest an interaction between the enoyl-ACP reductase, the last functional enzyme of FAS II complex and SAD [27]. In this context, the availability of purified SAD and thioesterase proteins would be very helpful to approach further studies.

In this work, we present the cloning and expression of genes encoding sunflower SAD and FatB in *E.coli* in order to characterize in the future the enzymatic properties of these enzymes, study the interaction between subunits, obtain antibodies against them, and undertake the study of the interaction between different proteins in the ''de novo'' fatty acids biosynthetic pathway.

2. Experimental

2.1. Plant material and growth conditions

Sunflower (*Helianthus annuus* L.) plants of public line RHA-274, with normal fatty acid content, were used in this work. Plants were cultivated in growth chambers at 20/10 °C (day/night) temperature, with a 16-h photoperiod and a photon flux density of 300 $\mu mol\ m^{-2}\ s^{-1}$.

2.2. mRNA preparation and cDNA synthesis

Approximately 0.4 g of developing sunflower seeds, 15 days after flowering, were ground in liquid N_2 with a precooled sterile mortar and pestle. The mRNA was isolated using a MicroFastTrack Kit (Invitrogen, Groningen, The Netherlands). The mRNA pellet was resuspended in 33 μl TE buffer (10 m*M* Tris–HCl, 1 m*M* EDTA, pH 8) and cDNA was obtained using a Ready-To-Go T-Primed First-Strand Kit (Amersham Bioscience, Roosendaal, The Netherlands).

2.3. Primers design

The SAD and FatB protein sequences available from databases were aligned using the Clustal X v1.8 program [28] in order to identify homologous regions. Two degenerated primers, containing flanking *Sph*I and *Xma*I sites, were designed from SAD highly conserved regions: SphSDS1 (5′-AAGCATG-CATGGCGMTTCGMATSARTMCGG-3′; 32 times degenerated) and XmaSDS2 (5′-TTCCCGGGTCA-GAGCTTCACTTCTCTATCRAARATCC-3′; four times degenerated).

In the case of the gene encoding FatB, we found, using the network based method, ChloroP for identifying chloroplast transit peptides [29], that Leu88 in sunflower FatB sequence was the best candidate for the N-terminal amino acid of the mature protein. Therefore, primers with additional restriction sites, *Sac*I and *Kpn*I, were designed in order to amplify the coding region for the mature protein by PCR: SacITioB1-2 (5′-GAGCTCCTGGAATGGAAGAC-CAAACGC-3′) and KpnITioB2 (5′-GGTACCTTA-AACATTTCCAGC-3′). Primers were synthesized by Amersham Bioscience (Roosendaal, The Netherlands).

2.4. Cloning

A 100 μl PCR reaction containing 10 m*M* Tris–HCl (pH 8.3), 50 m*M* KCl, 1.5 m*M* $MgCl_2$, 200 μ*M* of each deoxynucleotide triphosphate, 5U ECOTAQ DNA Polymerase (Hoffman-La Roche, New Jersey, USA), 100 pmol of each primer and single-stranded cDNA were used. All PCR amplifications were performed on a Clinus Microcycler 96 thermocycler using the following procedure: initial denaturation step, 96 °C for 5 min, 35 cycles with denaturation at 95 °C for 1 min, annealing at 55 °C for 1 min, extension at 72 °C for 1 min, followed by a final extension at 72 °C for 5 min. PCR fragments with the expected sizes were agarose-gel-purified (low melting type 14%) and extracted using Gelase (Epicentre). The PCR fragments were sequenced by GATC Gmbh (Konstanz, Germany) and the identity confirmed using Blast.

The PCR products were cloned into the *Sph*I-*Xma*I or *Sac*I-*Kpn*I sites of pQE30 (Qiagen, Chatsworth, CA), resulting in a fusion protein which carries six His amino acid residues as a tag at the N terminus. Ligation in the correct reading frame was confirmed by sequencing. The recombinant plasmids were introduced and expressed in *E.coli* strain BL21(pREP4), a BL21 strain which also carries the plasmid pREP4 containing the lac I^q repressor, in order to avoid toxic effect. BL21(pREP4/pQE30) strain was used as control.

2.5. Protein expression and purification

E. coli cells harboring recombinant plasmids, pQESADr (stearoyl-ACP desaturase) or pQEFATB (FatB thioesterase), were grown under continuous shaking at 37 °C in LB broth containing ampicillin (100 mg/l) and kanamycin (50 mg/l). The cells were induced at OD_{600} 0.5 with 0.4 m*M* isopropyl-β-D-thiogalactoside, and grown for an additional 4 h at 37 °C (or at 30 °C in the case of FatB). Bacterial cells were harvested by centrifugation (16 000 *g*) and resuspended in binding buffer (20 m*M* sodium phosphate, 0.5 *M* sodium chloride, 10 m*M* imidazole, pH 7.4). The cells were lysed by grinding with liquid N_2 in a mortar and the extract was centrifuged at 7000 *g*. The resulting soluble proteins were filtrated through 0.45 μm filters and loaded onto a Ni^{2+}-charged HiTrap Chelating HP 1 ml column interfaced with a Äkta*prime* system (both from Amersham Bioscience, Roosendaal, The Netherlands). The column was saturated with Ni^{2+} ions by using a 0.1 *M* $NiCl_2$ water solution according to the manufacturer instructions. Histidine-tagged proteins were eluted from the column using an imidazole gradient ranging from 10 to 500 m*M*. The subsequent wash and elution steps were performed at a flow-rate of 1 ml/min. During the washes and specific elution steps, the absorbance at 280 nm was monitored and fractions of 15 ml and 1 ml, respectively, were collected. The column was regenerated by washing with 500 m*M* EDTA in PBS, which removed all Ni^{2+} ions, and re-used. This repeated use had no effect on the reproducibility of the experiments. The enriched enzymes were exchanged into a buffer consisting of 20 m*M* sodium phosphate, 0.15 m*M* NaCl (pH 7.0), by gel filtration using a

Hi-Trap Desalting column (Amersham Bioscience). The proteins were stored in aliquots at −80 °C until use in enzymatic assays.

2.6. Gel electrophoresis

Samples for electrophoresis were combined with SDS–PAGE loading buffer and heated at 70–100 °C for 10 min. The samples were electrophoresed on 4–12% NU–PAGE gels in MES buffer (Invitrogen, Groningen, The Netherlands) and then fixed and stained in 1% Coomasie blue/45% methanol/10% acetic acid. Broad range molecular mass standard was purchased from Bio-Rad (Hercules, CA, USA).

2.7. Lipid extraction and analysis

Cultures (50 ml) of *E.coli* BL21 harbouring pQESADr, pQEFATB or a control plasmid without insert were grown to OD_{600} 0.9, then cells were harvested by centrifugation (5 min, 10 000 *g*) and resuspended in 3 ml of methanol:toluene:H_2SO_4 (88:10:2, v/v). Fatty acid methyl esters were obtained from lipids by heating the samples at 80 °C in 3 ml of methanol:toluene:H_2SO_4 (88:10:2, v/v) for 1 h [30]. After cooling, 1 ml of heptane was added and mixed. Fatty acid methyl esters were separated on a SP2380 capillary column (30 m long, 0.32 mm I.D., 0.20 μm film thickness) of fused-silica (Supelco, Bellefonte, PA, USA) and quantified by hydrogen flame ionization detection using a model 5890A gas chromatograph (Hewlett-Packard, Palo Alto, CA, USA). Hydrogen was used as carrier gas, with a linear gas rate of 28 cm s^{-1}. The injector and detector temperatures were 220 °C, oven temperature was 170 °C and the split ratio was 1:50. Fatty acids were identified by comparison with a combination of known standards and results from mass spectrometry of unknown peaks.

Samples for mass spectrometry were incubated with DMDS as described in [31]. A Carlo Erba GC8000 series gas chromatograph coupled with an MSD800 model (UG Analytical, Manchester, UK) was used for mass spectrometry analyses. A J&W DB-WAX fused-silica capillary column (30 m×0.25 mm I.D., 0.25 μm film thickness) was employed. The column temperature was held at 180 °C for 5 min, and then increased to 240 °C at 2 °C min^{-1}. The carrier gas (helium) flow-rate was 1 ml min^{-1}. The end of the fused-silica column was inserted directly into the ion source block. The spectra were recorded at an ionisation voltage of 70 eV and an ion source temperature of 200 °C. Mass range from 50 to 600 Da in full scan was selected.

3. Results and discussion

3.1. Isolation and sequence analysis of stearoyl-ACP desaturase and FatB thioesterase cDNAs

To clone and characterize the stearoyl-ACP desaturase and FatB thioesterase present in sunflower RHA-274 developing seeds, mRNA was isolated from frozen seed tissue and used for first-strand cDNA synthesis. The resulting cDNA was amplified by PCR using the oligos designed for SAD and FatB genes (Experimental 2.3). 1.2 Kb and 950 bp PCR products were obtained for sunflower SAD and FatB, respectively. In order to produce and purify considerable amounts of SAD and FatB proteins, the PCR products were purified and cloned into pQE30 vector, using the restriction sites incorporated at both ends of the oligos, obtaining the plasmids pQESADr, carrying SAD cDNA, and pQEFATB, carrying FatB cDNA. This plasmid, pQE30, allows IPTG-controlled expression of recombinant proteins in *E. coli* as N-terminally $His_{(6)}$-tagged fusion proteins.

The inserts were sequenced and shown to be homologous, with a 99% identity, to sunflower SAD and FatB genes previously described by Knapp et al. (GenBank sequences U91340 and AF036565, respectively). As shown in Fig. 1, the insertion of the modified cDNAs in the pQE30 vector would render proteins with additional amino acids with respect to the sunflower enzymes. Recombinant proteins bear the following N-terminal extensions: MRGSHHHHH-HGSA(SAD); and MRGSHHHHHHGSACE(FatB).

3.2. Expression and purification of stearoyl-ACP desaturase and FatB thioesterase

In order to purify recombinant proteins, *E. coli* BL21(pREP4) harbouring the pQESADr or pQEFATB plasmids were grown in rich medium supplemented with ampicillin and kanamycin to an

Fig. 1. Schematic diagram of the polylinker region in expression plasmids and the expected amino terminal sequence. (i) pQE30, vector designed for expression of 6× His-tagged proteins, underlined ATG indicates the start of the open reading frame (ORF) and boxes indicate the used restriction sites; (ii) pQESADr is a pQE30 expression vector containing the *Helianthus annuus* stearoyl-ACP desaturase cDNA (SAD) inserted at the *Sph*I/*Xma*I sites; (iii) pQEFATB is the expression vector containing de *Helianthus annuus* FatB thiosterase cDNA (FATB) (lacking the first 342 nucleotides of the coding region) inserted at the *Sac*I/*Kpn*I sites. Additional information from the expected fusion proteins were obtained with DNATools program (http://www.dnatools.dk).

optical density at 600 nm of 0.5 and expression of recombinant proteins was then induced by addition of 0.4 m*M* IPTG and incubation at 37 °C for an additional 4 h (pQESADr), or at 30 °C, also for an additional 4 h (pQEFATB). The cells overexpressing the recombinant proteins were disrupted with liquid nitrogen, and the proteins were recovered from the supernatant fraction. The proteins were purified by Immobilized Metal Affinity Chromatography (IMAC). Two 1 ml columns containing imino-diacetic acid chelating group charged with nickel ions (HiTrap Chelating) were used to purify the recombinant proteins. Commonly used protocols for IMAC purification of His-tagged proteins were applied, with washing steps that included different concentrations of imidazole and NaCl. Wash buffers containing a relatively low imidazole concentration (10 m*M*) resulted in elution of no specifically bound proteins. His-tagged enzymes were eluted with an increasing imidazole gradient (10–500 m*M*). His-SAD and His-FATB, eluted as a simple peak at 200 or 270 m*M* imidazole, respectively (Fig. 2). The fractions containing the recombinant proteins included some minor contaminating proteins.

The Coomassie blue-stained SDS–PAGE gels of these purified proteins are shown in Fig. 3 panels A and B. Both enzymes, His-SAD and His-FatB, were recovered at approximately 90% purity. Bands corresponding to an apparent molecular mass of about 45 000 were observed for both purified proteins, His-SAD and His-FATB, as determined from a plot of log molecular mass vs. Rg, the relative mobility. While these results are in agreement with the predicted His-SAD Mr (46 600 calculated for the 410-residue polypeptide), the expected value for His-FATB (37 400 for the 332 residues) was lower than the observed. This behaviour on His-FATB could be due to incomplete denaturation, due to the presence of long stretches of hydrophobic residues in this protein. As it has been previously reported that the most characteristic feature of the FatB thioesterases is the presence of a hydrophobic region in the N-terminus of mature proteins, after the transit peptides, which is absent in all FatA thioesterases [32].

3.3. Toxic effect of the expression of FatB thiosterase on E. coli cells

Initial attempts to obtain BL21 *E. coli* transformants containing pQEFATB plasmid were unsuccessful. To avoid a possible toxic effect, BL21 *E. coli* cells were previously transformed with plasmid pREP4 that includes the lac I^q repressor and would reduce expression from plasmid pQEFATB in the absence of IPTG. This strategy allowed us to obtain transformants harbouring our construct. Correct insertion of the FatB PCR fragment and appropriated frame were checked by sequencing. As shown in Fig.

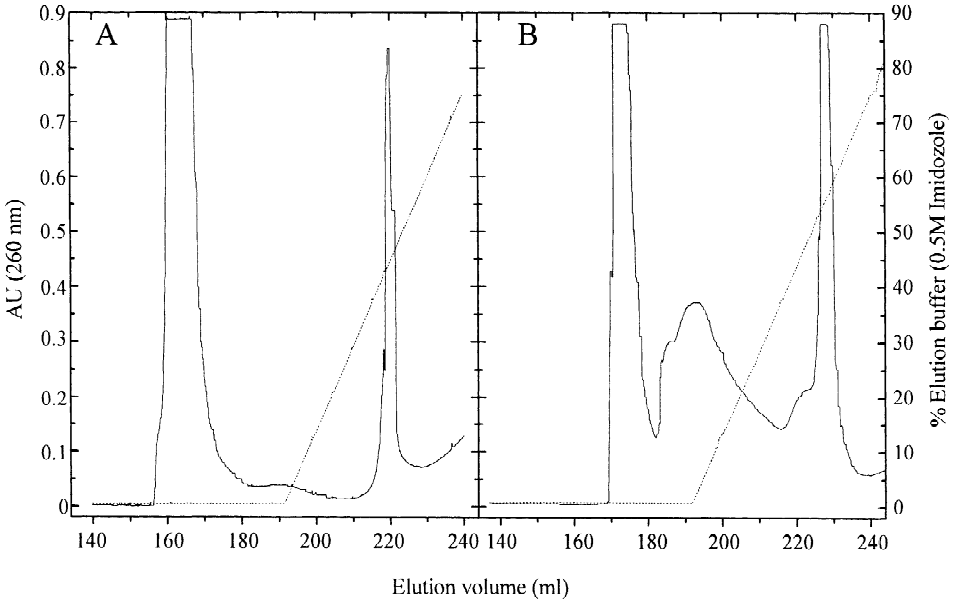

Fig. 2. Elution pattern of the purification of recombinant proteins by Immobilized Metal Affinity Chromatography (IMAC) with an Äkta*prime* system. The histidine-tagged stearoyl-ACP desaturase was eluted at 200 m*M* imidazole (A) while the histidine-tagged FatB thioesterase was eluted at 270 m*M* imidazole (B).

4, the expression of sunflower FatB thioesterase in *E. coli* resulted in a decreased growth rate. During initial logarithmic growth, no difference between cells expressing FatB and control cells was observed.

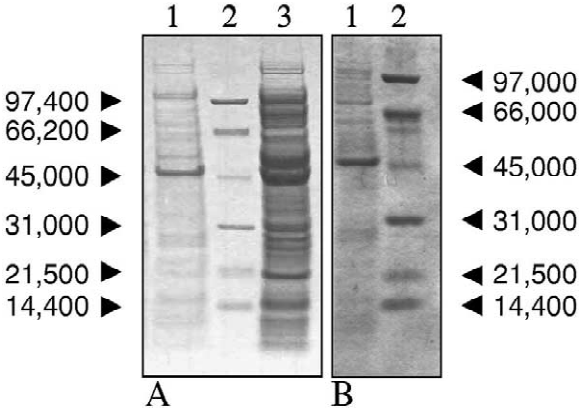

Fig. 3. Analysis by SDS–PAGE of fusion proteins, His-SAD (A) and His-FATB (B). Coomassie-blue-stained gel; lanes 1A and 1B, 40 μg and 20 μg eluted $(His)_6$ protein pool from bacterial expression of the recombinant sunflower stearoyl-ACP desaturase and FatB thioesterase, respectively; lanes 2A and 2B, molecular-mass standard; lane 3A, protein extract from *E. coli* control strain.

However, once IPTG was added our transformed culture reduced its growth rate entering into an early late log and stationary stages. Growth rate in induced cells was at least 2–10-fold smaller than in the corresponding control cells. This result suggests that FatB expression might affect aspects of cell metabolism, resulting in toxic effects to the bacterial cells, but it is unclear whether this is due to the accumulation of the recombinant protein or to the release of

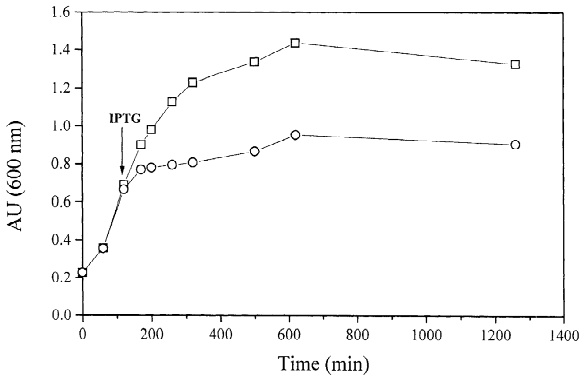

Fig. 4. Growth curve of *E. coli* control (□) and pQEFATB transformed (○) strains. IPTG was added at 120 min.

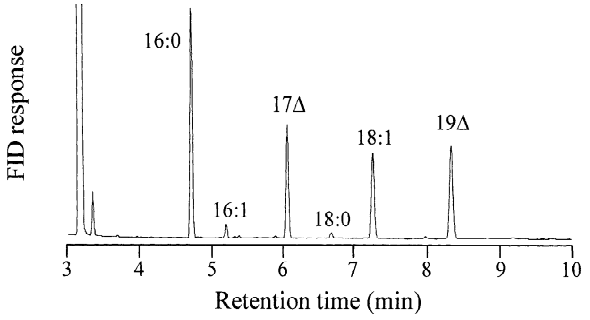

Fig. 5. Fatty acid profile from a stationary-phase culture of *E. coli* control strain BL21 harbouring pQE30 and pREP4 plasmids at 30 °C. Fatty acids: 16:0, palmitic acid; 16:1, palmitoleic acid; 17Δ, *cis*-9,10-methylenehexadecanoic acid (C17 CFA); 18:1, *cis*-vaccenic acid; 19Δ, *cis*-11,12-methyleneoctadecanoic acid (C19 CFA). CFA are formed by methylation of the UFAs palmitoleic and *cis*-vaccenic acids. While 16:0, 16:1 and 18:1 were identified with known standards, the cyclopropane derivatives, 17Δ and 19Δ, were characterized by mass spectrometry.

excess free fatty acids. Because free fatty acids are not normally found in *E. coli* as intermediates of lipid biosynthesis [20], it is possible that the expression of acyl-ACP thioesterases in *E. coli* increases the amounts of free fatty acids, that exhibit detergent properties, to toxic levels.

3.4. Fatty acid analysis of E. coli cells expressing recombinant proteins

Fatty acid profile from IPTG induced cultures of *E. coli* strainsBL21(pREP4/pQESADr), BL21(pREP4/pQEFATB) and control BL21(pREP4/pQE30) were obtained to test the functionality and effect of recombinant proteins on the *E. coli* lipid biosynthetic pathway (Fig. 5 and Table 1). *E. coli* cells expressing the sunflower SAD gene showed a reduction in the unsaturated/saturated fatty acids ratio mostly due to a slight increase in saturated fatty acids, and an increase in 16:0 derivatives, palmitoleic acid (16:1) and 17Δ, *cis*-9,10-methylenehexadecanoic acid (17:0 cyclopropane fatty acid (CFA)) This behaviour is similar to that found in *E. coli* strains with an increased expression of the *fab*A gene [33], responsible for the introduction of the *cis* double bound in bacterial fatty acid biosynthesis. These results suggest that expressed sunflower SAD is functional and interfering in the normal fatty acid biosynthetic pathway. Since there is a low amount of stearic acid (18:0) in bacteria [34] SAD seems to use palmitic acid (16:0) as an alternative substrate, as it has been previously described in high palmitic sunflower mutants, where higher amounts of 16:0 conduce to the accumulation of its derivative palmitoleic acid (16:1) due to SAD activity [35].

Whereas SAD expression shows clear effects, the expression of sunflower FatB thioesterase only shows an unbalance between different unsaturated fatty acids, a behaviour that has been observed previously when expressing FatB thioesterase from *Umbellularia californica* in *E. coli* [20]. This change to a higher amount of *cis*-vaccenic acid (18:1) can also be induced by growing *E. coli* at lower temperature, as a mechanism for maintaining proper membrane fluidity. As temperature has been constant in our experiment, the shift to vaccenate must be due to the interference of sunflower FatB thioesterase with the bacterial biosynthetic pathway. Additionally, the increase of *cis*-vaccenic acid does not correlate with an increase in 19Δ, *cis*-11,12-methyleneoctadecanoic

Table 1
Fatty acid composition of *E. coli* strains bearing control and recombinant plasmids. Strains were grown on rich medium supplemented with ampicillin and kanamycin until mid-log phase and the expression of recombinant proteins induced by IPTG addition

	T (°C)	Fatty acids (mol%)						UFA/SFA[b]	18:1/16:1[c]
		16:0	16:1	17:0[a]	18:0	18:1	19:0[a]		
Control	37	48.67	13.70	13.24	1.08	20.12	3.19	1.01	0.87
pQESADr	37	51.85	14.66	17.09	0.61	13.95	1.83	0.91	0.50
Control	30	36.52	2.03	20.47	1.13	17.57	22.29	1.66	1.77
pQEFATB	30	36.12	1.98	19.20	1.25	26.20	15.26	1.68	1.96

[a] Cyclopropane derivatives from unsaturated fatty acids, 17:0Δ and 19:0Δ.
[b] UFA, unsaturated fatty acid; SFA, saturated fatty acid.
[c] 18:1, includes 18:1 and its cyclopropane derivative 19Δ; 16:1, includes 16:1 and its cyclopropane derivative 17Δ.

acid (19:0 CFA) suggesting that part of this vaccenate accumulation is not been used in phospholipid biosynthesis and escapes the action of the cyclopropane fatty acid (CFA) synthase.

4. Conclusions

The expression of His-SAD or His-FatB produced changes in the *E.coli* fatty acid profile indicating the functionality of the recombinant proteins. While the expression of His-SAD produced an effect similar to that produced by overexpression of the FabA protein, responsible for the fatty acid desaturation in *E.coli*, the expression of His-FATB produced an unbalance between unsaturated fatty acids that results in a toxic effect in *E.coli*.

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