Research Article

Improved characterization of polyunsaturated fatty acids desaturases and elongases by co-expression in *Saccharomyces cerevisiae* with a protozoan acyl-CoA synthetase†

Running title: Coexpressing Acyl-CoA synthetase and PUFA desaturase

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Abstract

Saccharomyces cerevisiae is a valuable host for the expression and characterization of eukaryotic enzymes involved in polyunsaturated fatty acid (PUFA) biosynthesis, such as elongases and desaturases. The yeast allows a correct subcellular localization of these proteins, provides electron donors required by desaturases and is unable to synthesize PUFA that could interfere in the enzymes characterization. Unfortunately, *S. cerevisiae* incorporates very long chain PUFAs inefficiently, which could interfere in the characterization of enzymes using these substrates. Acyl-CoA synthetases (ACS) are involved in fatty acids uptake, and catalyze the synthesis of the corresponding CoA thioesters. ACS provides the substrates for elongases, acyl-CoA desaturases and acyl transferases. Transferases are required to synthesize phospholipids which in turn, are substrates for acyl-lipid desaturases. Expression in yeast of *Trypanosoma brucei* ACS1 notably improved the uptake of a wide variety of PUFA. Co-expression of ACS1 with Elo5 elongase from *Leishmania major* or Des4 desaturase from *T. brucei* showed respectively, 2 and 5.6 fold increases in the uptake of the PUFA substrates and 2.4 and 3.5 fold increases in substrate conversion. It also allowed to produce significant amount of Des4 desaturase product for further analysis, whereas it was obtained in trace amounts when the enzyme was expressed alone.

Practical applications: In this report, the use of yeast strains expressing ACS1 is proposed as a useful tool in the characterization of polyunsaturated fatty acids desaturases and elongases. Furthermore, this model could be used for the production of nutraceutical PUFA.

Abbreviations: ACS, acyl-CoA synthetases; **AA**, arachidonic acid; **BHT,** 2,6-di-tert-butyl-p-cresol; **DHA**, docosahexaenoic acid; **DPA**, docosapentaenoic acid; **DTA**, docosatetraenoic acid; **EPA**, eicosapentaenoic acid; **FAME**, fatty acid methyl esters; **GC**, gas chromatography; **GCMS,** gas chromatography coupled to mass spectrometry; **PUFA**, polyunsaturated fatty acids;

1 Introduction

Unsaturated fatty acids are essential components of biological membranes. They determine their structure and functions. Polyunsaturated fatty acids (PUFA) are also precursors of biologically active molecules like prostaglandins, leukotrienes and tromboxanes, which mediate a number of important physiological functions (i.e. fever, inflammations, vasodilatation). Long and very long chain PUFA, like arachidonic acid (AA, 20:4$\Delta^{5,8,11,14}$) and docosahexaenoic acid (DHA, 22:6$\Delta^{4,7,10,13,16,19}$), are the main fatty acids found in brain tissues [1, 2]. The fatty acid composition of mammalian cell membranes is greatly dependent on the dietary intake, which provides these PUFA or their precursors for endogenous synthesis. In the last 30 years the commercial use of vegetable and fish oils and, more recently, the use of alternative sources for oils with homogeneous and specific composition of PUFA as dietary supplements, has been recommended. This has led to the use of microorganisms such as fungi and protists in the search for new enzymes involved in PUFA biosynthesis as potential tools in the engineering of organisms with an optimized PUFA composition [3].

The biosynthesis of PUFA involves the introduction of double bonds (desaturation) in the aliphatic chain of a fatty acid and the extension (elongation) by two carbon units, of the acyl chain. It implies the alternate action of enzymes currently named desaturases and elongases, present in the endoplasmic reticulum of eukaryotes. Fatty acid substrates are in the form of thioesters like acyl-CoA, for elongases and some desaturases, or esterified to phospholipids for acyl-lipid desaturases. The mechanism of desaturation involves the use of molecular oxygen and reducing equivalents obtained from an electron transport chain [3].

Functional characterization of new elongases and desaturases usually implies heterologous expression in *Saccharomyces cerevisiae*, which lacks PUFA, but provides a good scaffold for expression of eukaryotic enzymes. Elongases and desaturases are correctly localized at the endoplasmic reticulum of the yeast, where the endogenous electron donor (usually compatible with the heterologous desaturase) is also available. These enzymes can be easily characterized by providing specific PUFA substrates to the transformed yeast followed by profiling the fatty acid composition. An important limitation of this system is the inefficiency of *S. cerevisiae* in the uptake of very long chain PUFA [4].

Fatty acids can cross the plasma membrane by passive diffusion or can be translocated by membrane associated transport proteins. Internalized fatty acids are activated to their CoA thioesters by acyl-CoA synthetases (ACS), trapping them inside the cell. This acylation guarantees a vectorial transport. *S. cerevisiae* have several ACS, all specialized in the activation of saturated or monounsaturated fatty acids no longer than 20 carbons [4]. The yeast is also able to synthesize de novo saturated fatty acids up to 26 carbons and medium chain monounsaturated fatty acids, like palmitoleic (16:1) and oleic (18:1) acids [5]. By contrast, protists membranes usually contain a high proportion of PUFA [3].

We have previously described the PUFA biosynthetic pathways present in flagellated protozoa of clinical interest belonging to the genera *Trypanosoma* and *Leishmania*. This has led us to deal with the expression in yeast of trypanosomatid elongases and desaturases which required the use of PUFA like linolenic acids (18:3), AA, docosapentaenoic acid (DPA) or docosatetraenoic acid (DTA) as substrates [6-8]. The low incorporation of several of these PUFA have hampered the complete characterization of some enzymes, as the corresponding products usually appeared at the limit of detection, even using sensitive technologies like gas chromatography coupled to mass spectrometry (GCMS). Trypanosomatids also contain multiple ACS with different substrate specificities. For instance, *Trypanosoma brucei* ACS1 was described as being able to activate a wide variety of PUFA [9, 10]. Here we report that expression of ACS1 notably increased the incorporation of long and very long PUFA in yeast. Co-expression of ACS1 with elongases or desaturases, improved the amount of the enzyme products in the fatty acid profile of the yeasts allowing their unequivocal identification and, as a consequence, the correct characterization of these enzymes.

2 Materials and Methods

2.1 Materials

Fatty acids, Tergitol (type Nonidet P-40), sodium methoxide, ampicillin, yeast nitrogen base, glucose, amino acids and 2,6-di-tert-butyl-p-cresol **(**BHT) were obtained

from Sigma (Sigma-Aldrich, St. Louis, MI, USA). All organic solvents were purchased from Merck (Whitehouse Station, NJ, USA).

2.2 Cloning, sequencing and sequence analysis

T. brucei ACS1 gene sequence was retrieved from the database of the trypanosomatid genome projects (TriTrypDB, http://www.tritrypdb.org), based on information from Jiang and Englund. [9]. The sequence (locus Tb927.9.4190) was analyzed using tools available online (http://www.genedb.org and http://www.ncbi.nlm.nih.gov). Procyclic *T. brucei* (strain 427) cells were grown in SDM-79 medium supplemented with 10 % fetal bovine serum and hemin [11]. Genomic DNA was prepared by standard methods. Primers Ac1, 5'-CGGGATCC**ATG**TACGTCACTAAGCTTCTCTCC -3' and Ac2, 5'- CCCTCGAG**TTA**ATGATGATGATGATGATGCTCATACTTGAAAATTGTTCAA -3' were designed to amplify the gene. The underlined sequences represent BamHI and XhoI sites; oligonucleotides also include the natural initiation and stop codons (in bold). Amplification was carried out in 50 μL under the following conditions: initial incubation at 94 °C for 4 min, followed by 30 cycles of denaturation at 94 °C for 40 s, annealing at 56 °C for 40 s and extension at 68 °C for 10 min. Amplified fragments were cloned using pGEM-T Easy vector (Promega, Madison, WI, USA), according to the manufacturer's procedure, and used to transform competent *Escherichia coli* (XL1-Blue). Plasmids purified from positive clones were sequenced completely.

2.3 Expression of cloned genes

The cloned sequence was ligated into the BamHI and XhoI sites of p425GPD, a yeast expression vector, containing the glyceraldehyde-3-phosphate dehydrogenase constitutive promoter [12]. A selectable marker gene in this vector confers leucine prototrophy to the host. Either the vector alone, or the vector harbouring ACS1, was used to transform *S. cerevisiae* strain HH3 (MAT*a*, trp1-1, ura3-52, ade2-101, his3-200, lys2-801, leu2-1) [13] by electroporation. Transformed clones were selected on minimal agar plates lacking leucine. The obtained yeast lines (Sc-p425 and Sc-ACS1) were also transformed with p426GDP (conferring uracil prototrophy) [12] alone or harbouring the *T. brucei* Δ4 desaturase gene Tb927.10.7100 [7] (lines Sc-Des4 and Sc-ACS1-Des4) or

the *Leishmania major* Elo5 elongase gene LmjF.05.1170 [8] (lines Sc-Elo5 and Sc-ACS1-Elo5).

In order to determine the incorporation of exogenous fatty acids and enzyme activities (substrate conversion), transformed yeasts were cultured for 48 h at 30 °C in 0.67 % (w⁄v) yeast nitrogen base, 2 % (w⁄v) glucose and tryptophan, lysine, adenine and histidine (all at 20 mg/mL). Cultures were diluted to an OD of 0.2 at 600 nm, supplemented with fatty acids and grown for 72 h at 30 °C with constant agitation. Fatty acids were prepared in ethanol containing BHT (50 ppm) at a stock concentration of 70 mM and added to a final concentration of 70 or 7 μM into 20 mL cultures containing 0.2 % (v⁄v) Tergitol.

2.4 Fatty acid analysis

Twenty mL cultures were centrifuged at 5000 *g* for 5 min, and pelleted cells were washed twice with an equal volume of distilled water. Lipids were extracted as described by Bligh and Dyer [14]. The organic phase was dried under N_2 and fatty acid methyl esters (FAME) were obtained by incubation with 1 mL of 0.5 M sodium methoxide in methanol for 20 min at room temperature. Following neutralization with 6 M HCl and extraction with 2 mL of hexane, the solvent was evaporated to dryness under a N_2 atmosphere and dissolved in isohexane containing BHT for GCMS analysis.

The composition of FAME was analysed by gas chromatography (GC) running samples through an SE-30 column (25 m × 0.22 mm inside diameter; Scientific Glass Engineering, Ringwood, Victoria, Australia) in a Shimadzu GC-2010 Plus gas chromatograph (Shimadzu Corp., Kyoto, Japan). Column temperature was programmed to increase from 170 to 240 °C at a rate of 3.5 °C/min. Helium was the carrier gas at a constant flow rate of 1 mL/min. The GCMS was carried out using a GCMS-QP2010 Plus detector (Shimadzu) operated at an ionization voltage of 70 eV with a scan range of 40–600 Da. The retention time and mass spectrum of any new peak obtained was compared with that of standards (Sigma) and those available in the data base NBS75K (National Bureau of Standards). Percentages of FAME were calculated after integration of the chromatogram peaks. Enzyme activity was estimated as percentage of total (substrate plus product) fatty acid substrate converted into the corresponding product. Results represent the mean ± SD values of three independent experiments. For double bond positional analysis,

dimethyloxazoline derivatives were prepared by adding 2-amino-2-methyl-1-propanol (Sigma) to the lipid sample and analysed as described [15].

3 Results and discussion

3.1 Expression of *T. brucei* ACS1 in *S. cerevisiae*

T. brucei contain five ACS, which were earlier expressed in *E. coli*, purified and characterized in vitro with respect to their substrate specificities [9, 10]. One of those (ACS1) showed a marked preference for saturated fatty acids of medium and long chain, and for long and very long chain PUFA [9]. This is in line with the needs of *Trypanosoma* sp. to take up PUFA from their hosts, which are incorporated directly or after desaturation and elongation, for the synthesis of AA and DHA [6]. ACS1 was cloned and expressed in yeast. The PUFA intake level of the yeast line harbouring the gene (Sc-ACS1) was compared to that of the corresponding control (Sc-p425) which carried the vector alone. This was performed by supplementing the media with a wide variety of fatty acids, one at a time, and analyzing their profiles by GCMS after 72 h of growth. Fig. 1 shows the percentage of the supplemented fatty acids incorporated by the yeasts cells obtained as FAME from total lipid extracts. The result of this in vivo approach is in agreement to the previous in vitro characterization of ACS1 [9], with a 1.6 to 4.2 fold increase in C20-PUFA intake. Additionally, assay of DTA (22:4$\Delta^{7,10,13,16}$) showed a remarkable 7.6 fold increased intake for Sc-ACS1. By contrast, intake of C18 PUFA, monounsaturated or saturated fatty acids were not significantly stimulated by ACS1, probably because the endogenous ACS activities were sufficiently high to saturate the yeast capacity to deal with these substrates (see also Fig. 2).

3.2 Effect of substrate concentration and temperature in the incorporation of PUFA by yeast

Both yeast lines were cultured at increased concentrations of PUFA at 30° C. As shown in Fig. 2, the uptake level was proportional to the concentration of the supplements. Interestingly, the intake of 18:3$\Delta^{9,12,15}$ by both cell lines showed a similar dependence on the external concentration of this substrate, which could be explained by the predominant

action of the endogenous ACS and a marginal activity of ACS1. By contrast, intake of AA appeared to be mainly conducted by the heterologous ACS1, in good agreement with its known substrate specificity.

The uptake of AA was also assayed at two temperatures and two substrate concentrations: one which is that more frequently utilized in this kind of assays (70 μM) and at 7 μM, in order to challenge the in vivo activity of ACS1 in yeast. Table 1 shows that PUFA intake was highly dependent of temperature for the control yeast line, with approximately a 2.8-fold increase in AA uptake at 20° C, irrespective of the supplement concentration. This observation is probably related to regulatory processes present in yeasts to maintain a correct membrane fluidity, which involves also the regulation of ACS activities [16]. Interestingly, Sc-ACS1 incorporated approximately the same amount of AA at both temperatures, being 4-fold higher than the control at 30 °C, at both supplement concentrations. This increased and stable (with respect to the culture conditions) incorporation of PUFA in Sc-ACS1, is desirable when choosing a correct host line for the expression of enzymes of PUFA metabolism. Furthermore, it also allows the use of lower amounts of substrates, which are usually expensive.

3.3 Co-expression of ACS1 with *Leishmania major* Elo5 elongase

L. major Elo5 catalyzes very efficiently the elongation of PUFA containing a double bond between carbons 5 and 6. It uses preferentially AA and eicosapentaenoic acid (EPA), 20:5$\Delta^{5,8,11,14,17}$), with substrate conversions of approximately 60 % and 37 %, respectively. However, Elo5 is able to unspecifically use other substrates, like 18:3$\Delta^{6,9,12}$, 18:4$\Delta^{6,9,12,15}$ and 20:3$\Delta^{8,11,14}$, with conversion ratios of 10.6, 5.6 and 3.6 %, respectively [8]. As acyl-CoAs are both the direct products of ACS and the substrates of elongases, Elo5 was chosen for co-expression with ACS1. This approach would allow an increase in the internal concentration of the supplemented PUFA thioester, that will serve as substrate for the augmented Elo5 activity. To challenge the method, the least efficient substrate (20:3$\Delta^{8,11,14}$) was used to supplement the culture medium of the co-expressing yeast line Sc-ACS1-Elo5 and the corresponding control Sc-Elo5. Table 2 shows that co-expression with ACS1 significantly increased the substrate intake and doubled its conversion ratio.

3.4 Co-expression of ACS1 with *T. brucei* Des4 desaturase

In order to test another kind of enzyme and substrates, ACS1 was co-expressed with *T. brucei* Des4 desaturase, which converts the very long chain PUFA 22:4$\Delta^{7,10,13,16}$ and 22:5$\Delta^{7,10,13,16,19}$ into 22:5$\Delta^{4,7,10,13,16}$ and DHA, with ratios of 4.4 % and 5.8 %, respectively [7]. As a consequence of these low conversion ratios, C22-PUFA products represented approximately a mere 0.05 % of total FAME in the GCMS analysis, which is the detection limit of the method. Table 3 shows that supplementing the culture media with 22:4$\Delta^{7,10,13,16}$, line Sc-ACS1-Des4 incorporated 5.6 fold more DTA than the control line (Sc-Des4), and the substrate conversion was 3.5 fold higher for the co-expressing line. This notable increase in the enzyme product, which represents 0.9 % of total FAME (Fig. 3), allowed its correct identification by direct analysis of its mass spectrum or that of its dimethyloxazoline derivative (not shown). These findings unambiguously confirm the enzyme characterization as a PUFA Δ4 desaturase.

4 Conclusions

Expression in yeast of *T. brucei* ACS1 allowed significant increases in the uptake of exogenous PUFA. This increment was particularly high with C22-PUFA, which was essential in the unambiguous characterization of *T. brucei* Des4 desaturase. Co-expression of both enzymes allowed a remarkable increase in the incorporation of desaturase substrate and in the amount of product obtained. We propose the use of transformed yeasts expressing this ACS as a tool in the characterization of enzymes involved in PUFA metabolism. Other ACS, with different substrate preferences, may be used to introduce specific modifications in the fatty acid profile of yeasts according to the properties of the enzymes to be assayed. This strategy could be applied in the endogenous generation of a desired PUFA, as substrate for co-expressed enzymes or, as a final product with academic or nutraceutical applications.

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Tables:

Table 1. Temperature effect on the incorporation of 20:4$\Delta^{5,8,11,14}$. Yeasts were cultured during 72 h at the indicated temperatures at two substrate concentrations. Numbers indicate the percentage of 20:4$\Delta^{5,8,11,14}$ in total FAME. Between parentheses, the fold increase found in Sc-ACS1 line relative to the control line Sc-p425. SD of at least three independent determinations are shown.

[20:4$\Delta^{5,8,11,14}$]	**20 °C**		**30 °C**	
	Sc-p425	**Sc-ACS1**	**Sc-p425**	**Sc-ACS1**
70 μM	7.81±0.50	10.95±1.11 (1.4)	2.90±0.32	11.10±1.32 (3.8)
7 μM	0.90±0.11	1.51±0.30 (1.7)	0.31±0.10	1.32±0.20 (4.3)

Table 2. Incorporation in yeast lines of 20:3 and its conversion ratios by ELO5 elongase. Numbers indicate percentages of fatty acids relative to total FAME and percentage of 20:3 converted into 22:3. Between parentheses the fold increase in conversion ratio relative to the control line Sc-ELO5. SD of at least three independent determinations are shown.

	Sc-ACS1-Elo5	**Sc-Elo5**
20:3$\Delta^{8,11,14}$	17.71±1.22	9.59±1.13
22:3$\Delta^{10,13,16}$	2.19±0.40	0.46±0.15
20:3 → 22:3	11.01±1.00 (2.4)	4.60±0.33

Table 3. Incorporation in yeast lines of 22:4 and its conversion ratios by Des4 desaturase. Numbers indicate percentages of fatty acids relative to total FAME and percentage of 22:4 converted into 22:5. Between parentheses the fold increase in conversion ratio relative to the control line Sc-Des4. SD of at least three independent determinations are shown.

	Sc-ACS1-Des4	**Sc-Des4**
22:4$\Delta^{7,10,13,16}$	5.33±0.51	1.06±0.44
22:5$\Delta^{4,7,10,13,16}$	0.87±0.33	0.04±0.01
22:4 → 22:5	14.0±1.8 (3.5)	4.00±0.05

Figures and figure legends:

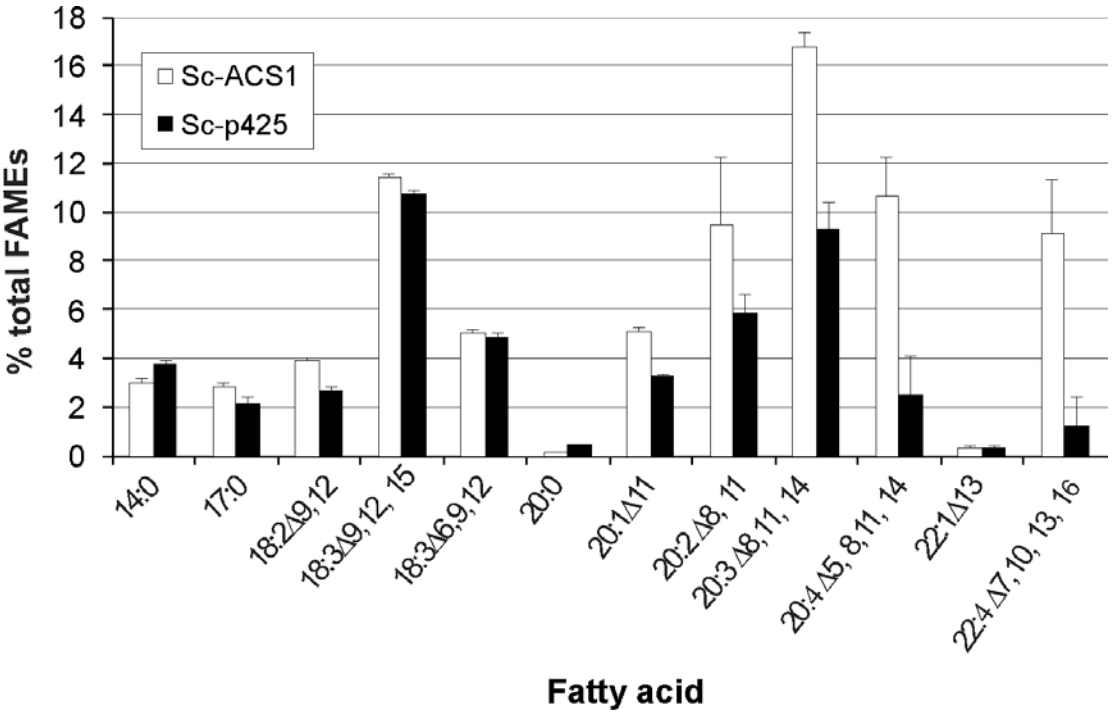

Figure 1. Comparison of the intake levels of exogenously added fatty acids in yeast expressing ACS1 (Sc-ACS1) or harbouring the empty vector (Sc-p425). Only a single fatty acid was added, at a concentration of 70 μM to the culture medium, in each experiment and the yeast cultured for 72 h when the FAMEs were measured. Values of incorporated FAs are expressed as percentages of total cellular FA. SD of at least three independent determinations are shown.

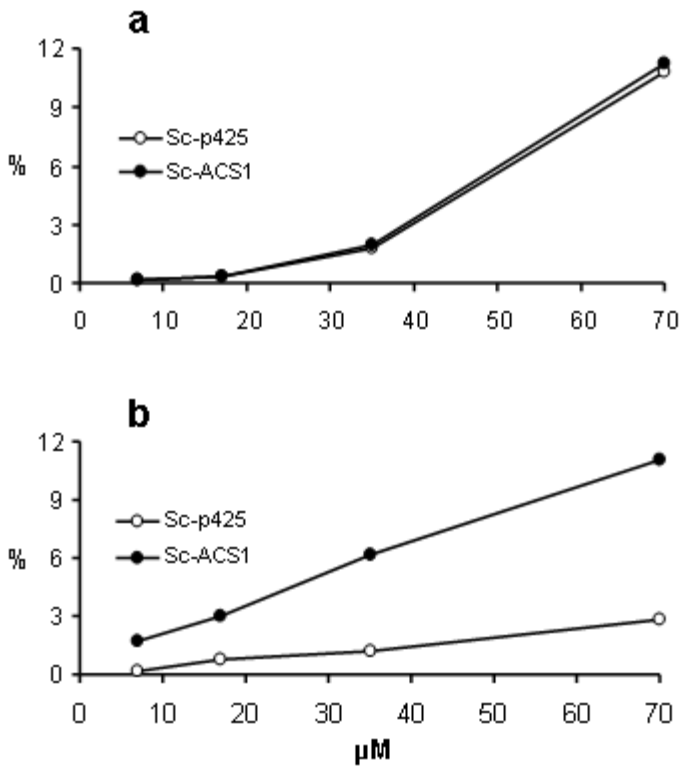

Figure 2. Intake of 18:3$\Delta^{9,12,15}$ (a) and 20:4$\Delta^{5,8,11,14}$ (b). Yeast lines were cultured during 72 h at 30 °C in the presence of the corresponding PUFA at the indicated concentrations. Total fatty acids were extracted and analysed by GCMS as FAME derivatives. Relative content of each supplemented fatty acid is expressed as percentage of total fatty acids in the extract. One representative of three similar experiments is shown.

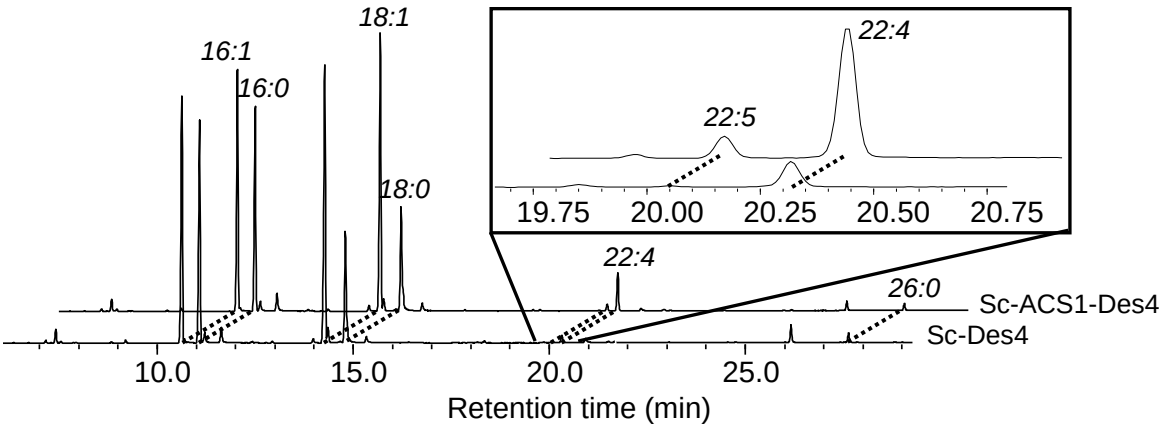

Figure 3. GC analysis of FAME obtained from total lipid extracts of Sc-Des4 and Sc-ACS1-Des4 yeast lines. Culture media were supplemented with 70 μM of 22:4$\Delta^{7,10,13,16}$. Inset shows a 10 × amplification of the region corresponding to retention times between 19.6 and 20.8 min. The main yeast fatty acids, as well as the substrate (22:4) and Des4 product (22:5) are indicated (in italics).