

Isolation of a $\Delta 5$ Desaturase Gene from *Euglena gracilis* and Functional Dissection of Its HPGG and HDASH Motifs

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Abstract Delta (Δ) 5 desaturase is a key enzyme for the biosynthesis of health-beneficial long chain polyunsaturated fatty acids such as arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (C20:5n-3) and docosahexaenoic acid (C22:6n-3) via the “desaturation and elongation” pathways. A full length $\Delta 5$ desaturase gene from *Euglena gracilis* (*Eg $\Delta 5D$*) was isolated by cloning the products of polymerase chain reaction with degenerate oligonucleotides as primers, followed by 5' and 3' rapid amplification of cDNA ends. The whole coding region of *Eg $\Delta 5D$* was 1,350 nucleotides in length and encoded a polypeptide of 449 amino acids. BlastP search showed that *Eg $\Delta 5D$* has about 39 % identity with a $\Delta 5$ desaturase of *Phaeodactylum tricornutum*. In a genetically modified dihomogamma-linoleic acid (DGLA, C20:3n-6) producing *Yarrowia lipolytica* strain, *Eg $\Delta 5D$* had strong $\Delta 5$ desaturase activity with DGLA to ARA conversion of more than 24 %. Functional dissection of its HPGG and HDASH motifs demonstrated that both motifs were important, but not necessary in the exact form as encoded for the enzyme activity of *Eg $\Delta 5D$* . A double mutant *Eg $\Delta 5D$ -34G158G* with altered sequences within both HPGG and HDASH motifs was generated and exhibited $\Delta 5$ desaturase activity similar to the wild type *Eg $\Delta 5D$* . Codon optimization of the

N-terminal region of *Eg $\Delta 5D$ -34G158G* and substitution of the arginine with serine at residue 347 improved substrate conversion to 27.6 %.

Keywords $\Delta 5$ desaturase · HPGG motif · HDASH motif · Double mutant · Fatty acid biosynthesis · *Yarrowia lipolytica*

Abbreviations

ALA	Alpha-linolenic acid (ALA 18:3n-3)
ARA	Arachidonic acid (C20:4n-6)
ATCC	American Type Culture Collection (Rockville, MD)
DAG	Diacylglycerol
Dd $\Delta 5D$	$\Delta 5$ Desaturase of <i>Dictyostelium discoideum</i>
DGLA	Dihomo-gamma-linoleic acid (C20:3n-6)
DHA	Docosahexaenoic acid (C22:6n-3)
DPA	Docosapentaenoic acid (22:5n-3)
Eg $\Delta 4D$	$\Delta 4$ Desaturase of <i>Euglena gracilis</i>
Eg $\Delta 5D$	$\Delta 5$ Desaturase of <i>Euglena gracilis</i>
Eg $\Delta 8D$	$\Delta 8$ Desaturase of <i>Euglena gracilis</i>
EG Media	<i>Euglena</i> growth media
FFA	Unesterified fatty acids
FOA	5-Fluoro-orotic acid FOA
EPA	Eicosapentaenoic acid (C20:5n-3)
ETA	Eicosatetraenoic acid (C20:4n-3)
GC	Gas chromatography
HGM	High glucose media
His-rich motif	Histidine-rich motif
Ig $\Delta 5D$	$\Delta 5$ Desaturase of <i>Isochrysis galbana</i>
LC-PUFA	Long chain polyunsaturated fatty acids
LNA	Linoleic acid (18:2n-6)
Ma $\Delta 5D$	$\Delta 5$ Desaturase of <i>Mortierella alpina</i>
MMLeu	Minimal media + leucine

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NL	Neutral lipids
Ot Δ 5D	Δ 5 Desaturase of <i>Ostreococcus tauri</i>
PCR	Polymerase chain reaction
Pi Δ 5D	Δ 5 Desaturase of <i>Pythium irregulare</i>
PL	Phospholipids
PI Δ 8D	Δ 8 Desaturase of <i>Pavlova lutheri</i>
Pm Δ 5D	Δ 5 Desaturase of <i>Phytophthora megasperma</i>
Pt Δ 5D	Δ 5 Desaturase of <i>Phaeodactylum tricornutum</i>
PUFA	Polyunsaturated fatty acids
RACE	Rapid amplification of cDNA ends
TAG	Triacylglycerol
Ta Δ 5D	Δ 5 Desaturase of <i>Thraustochytrium aureum</i>
Ts Δ 8D	Δ 8-Sphingolipid desaturase of <i>Thalassiosira pseudonana</i>

List of symbols

α	Alpha
γ	Gamma
Δ	Delta
#	Number

Introduction

There is increasing interest in the recognized health benefits of long chain polyunsaturated fatty acids (LC-PUFA) such as arachidonic acid (ARA, C20:4n-6), eicosapentaenoic acid (EPA, C20:5n-3) and docosahexaenoic acid (DHA, C22:6n-3) for both humans and animals [1–3]. Since mammals lack delta (Δ) 12- and Δ 15-desaturases, ARA, EPA and DHA cannot be synthesized de novo and must be obtained either in the diet or synthesized through “desaturation and elongation” pathways (Fig. 1) from essential fatty acids linoleic acid (LNA, 18:2n-6) and/or alpha (α)-linolenic acid (ALA 18:3n-3). These LC-PUFA are important fatty acids for human growth and development. For example, ARA, a precursor of EPA, is abundant in the brain and muscles. As a lipid second messenger ARA is involved in cellular signaling and is a key inflammatory intermediate [3]. EPA is a precursor of DHA, and induces a broad anti-inflammatory response [1–3]. DHA is a major ω -3 fatty acid in the mammalian central nervous system and enhances synaptic activities in neuronal cells [1–4]. EPA and DHA are the precursors of E- and D-series resolvins, respectively. These two classes of resolvins have distinct structural, biochemical and pharmacological properties [5, 6]. ARA and DHA together play critical roles for neurological development and health [4, 7]. Dietary EPA and DHA can effectively reduce the level of blood triglycerides in human [8]. Increased intake of EPA-rich

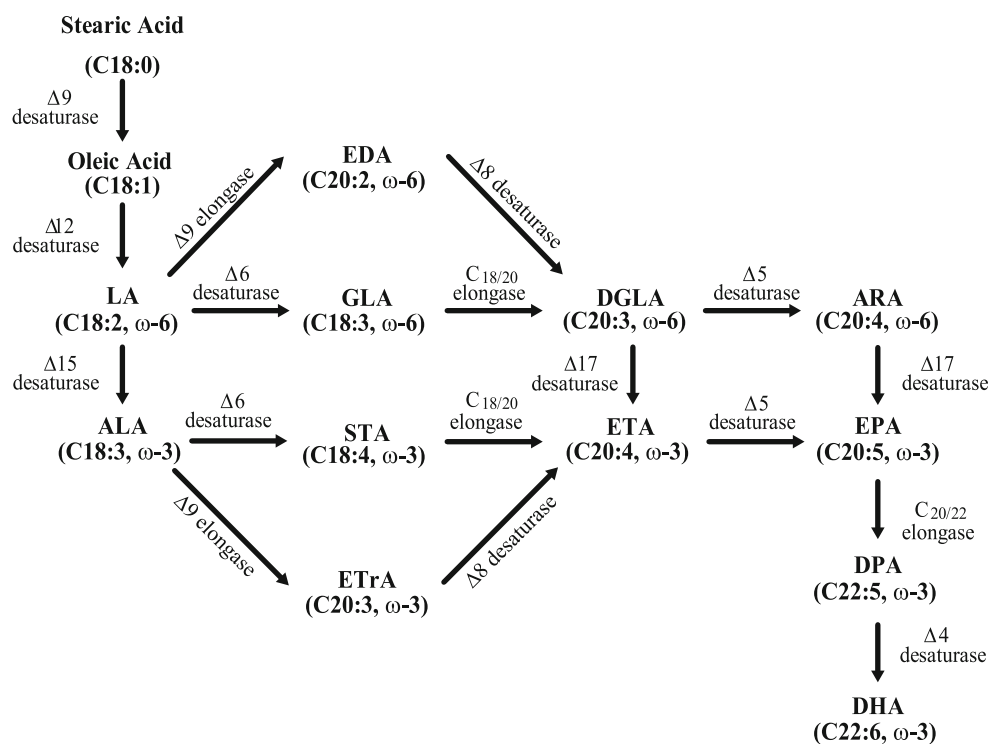
supplement has beneficial effects on coronary heart disease, high blood pressure, inflammatory disorders and mental illness [9, 10].

Currently, the primary source of EPA and DHA is marine fish oil. Most of the EPA and DHA in fish oil are from their cold-water oceanic microalgae food sources. More than 85 % of isolated fish oil is used for aquaculture. In the case of salmon-farming, fish oils from approximately 4 pounds of fish are needed to raise one pound of salmon filet, the fish-in and fish-out ratio is about 4:1. In today’s environment, wild-caught fish often contain contaminants such as methylmercury, polychlorinated biphenyls, dioxins and several other halogenated persistent organic pollutants [11]. With ever-growing human populations, and limited sources of ocean fish, there is growing concern about the quality, quantity and sustainability of fish oil.

In the last two decades, great efforts have been focused on developing different hosts for production of LC-PUFA. Wild type *Mortierella alpina* has been developed for commercial production of ARA [12], while *Cryptocodinium cohnii* and *Schizochytrium* have been developed for commercial production of DHA [13]. The ARA and DHA oils produced from these organisms have been largely used in infant formulas. *Yarrowia lipolytica* has been genetically engineered to contain an EPA biosynthesis pathway [14] allowing for the commercial production of EPA oil, NewHarvest™ (<http://www.newharvest.com>). The EPA oil has been used as a human nutritional supplement. Additionally, EPA-rich *Yarrowia* biomass has been used to feed a brand of farmed salmon, Verlasso™ (<http://www.verlasso.com>), with a fish-in and fish-out ratio of about 1:1. However, the current production scale and cost of ARA, EPA and DHA cannot meet the market demand.

Various organisms use different pathways to synthesize ARA, EPA and DHA. *Cryptocodinium* and *Schizochytrium* synthesize EPA and DHA through a polyketide-based pathway [15], while some species of algae, fungi, and protists synthesize ARA, EPA and DHA through fatty acid “desaturation and elongation” pathways [16, 17]. All “desaturation and elongation” pathways (Fig. 1) require LNA and/or ALA as a substrate, followed by the “ Δ 9 elongase and Δ 8 desaturase” pathway or the “ Δ 6 desaturase and C_{18/20} elongase” pathway to synthesize ARA, EPA and DHA by orchestrated elongation and desaturation reactions. So far, genetic engineering has mainly employed these “desaturation and elongation” pathways to modify hosts such as plants and yeast to produce ARA, EPA and DHA [14, 18–23]. Production of ARA, EPA and DHA through “desaturation and elongation” pathways requires gene expression of Δ 5 desaturase to catalyze the conversion of di-homo- γ -linolenic acid (DGLA, C20:3n-6) to ARA, with a similar activity of converting eicosatetraenoic acid (ETA, C20:4n-3) to EPA. Since the isolation of Δ 5

Fig. 1 Biosynthetic pathways for ARA, EPA and DHA via “desaturation and elongation” pathways



desaturase gene from *Mortierella alpina* (*MaΔ5D*, 24, 25), several $\Delta 5$ desaturase genes have been isolated [26], however, there is little research about their structure/function relationship. Additionally, more efficient $\Delta 5$ desaturase genes are needed to ensure engineered organisms may produce high levels of ARA, EPA and DHA.

$\Delta 5$ desaturases are known as “front-end” desaturases, wherein desaturation occurs between a pre-existing double bond and the carboxyl terminus of the fatty acid [26–29]. Like other desaturases, $\Delta 5$ desaturase is an iron-containing and membrane-bound enzyme that requires both molecular oxygen and an electron transfer to introduce double bonds into an existing acyl chain. Microsomal cytochrome b_5 serves as electron donor to desaturase enzymes [26, 29, 30]. Fatty acid desaturation can be carried out via concerted action of multiple enzymes including NADH reductase, the desaturase enzyme, and cytochrome b_5 reductase. Alternatively, many desaturases contain both a cytochrome b_5 domain and a desaturase domain. For example, $\Delta 4$, $\Delta 5$, $\Delta 6$ and $\Delta 8$ desaturases have a cytochrome b_5 domain at their N-terminus [26]; $\Delta 9$ desaturase has a cytochrome b_5 domain at its C-terminus [30]. The cytochrome b_5 domain of all desaturases has a heme-binding “HPGG” motif. Previous studies using molecular dynamics simulations suggest the “HPGG” motif is important for heme group assembly and desaturase function [31, 32].

The active site of desaturases has been characterized as a diiron cluster that is bound to the enzyme by three regions

of highly conserved histidine-rich (His-rich) motifs [27, 30]. These three His-rich motifs $H(X)_{3-4}H$, $H(X)_{2-3}HH$, and $H/Q(X)_{2-3}HH$ are conserved among all front-end desaturases, and the eight histidine residues of these three motifs are essential for catalytic activity [33]. In the case of $\Delta 5$ desaturase from *MaΔ5D* [24, 25], the exact amino acid sequence of the first His-rich motif ($H(X)_{3-4}H$) is HDASH, which has been suggested as one of the characteristics of $\Delta 5$ desaturases and necessary for its function to convert DGLA to ARA [34]. Recent studies find that several $\Delta 5$ desaturases (GenBank accession #: AAL82631, AAL13311, AAL92562, AAM09687, CAJ07076) do not contain the exact HDASH sequence. Due to the important role of $\Delta 5$ desaturases in LC-PUFA biosynthesis, a detailed understanding of the functional significance of the conserved HPGG and HDASH motifs may contribute to improvements in hosts biologically engineered to produce commercially valuable LC-PUFA.

We report the isolation of a $\Delta 5$ desaturase gene from *Euglena gracilis* (*EgΔ5D*). Expression of *EgΔ5D* in a genetically modified DGLA producing *Y. lipolytica* strain revealed that *EgΔ5D* had strong $\Delta 5$ desaturase activity. Functional dissection of HPGG and HDASH motifs demonstrated that neither the HPGG nor the HDASH motif is necessary in the exact form as encoded for enzyme activity of *EgΔ5D*. Various mutants, within HPGG or HDASH motif alone, or within both HPGG and HDASH motifs, are functionally equivalent or have higher $\Delta 5$ desaturase activity than the wild type *EgΔ5D*. Codon optimization of

the N-terminal region of a double mutant *EgΔ5D-34G158G* and substitution of the arginine with serine at residue 347 effectively improved the enzyme's substrate conversion.

Materials and Methods

Strains, Media and Growth Conditions

E. gracilis was kindly provided by Dr. Richard Triemer of Michigan State University (East Lansing, MI). *Euglena* growth (EG) media (per liter): 1 g sodium acetate, 1 g of beef extract, 2 g of Bacto® tryptone and 2 g of Bacto® yeast extract in 970 mL of water. After filter sterilizing, 30 mL of soil–water supernatant was aseptically added. A 1-mL aliquot of *E. gracilis* culture was transferred into 250 ml of EG Medium in a 500-mL glass bottle. The cultures were grown at 23 °C with a 16 h light, 8 h dark cycle for 2 weeks with no agitation.

Y. lipolytica strain Y2224 is a 5-fluoroorotic acid (FOA) resistant mutant of wild type strain American Type Culture Collection (ATCC, Rockville, MD) #20362 (Fig. 2a) with a mutation in the *URA3* gene (Genbank accession#: No. AJ306421). *Y. lipolytica* strain Y4036U (Zhu et al., unpublished data) is a genetically modified strain with a *Leu*⁻ and *Ura*⁻ phenotype, also originated from the wild type strain ATCC #20362. Strain Y4036U produced

approximately 18 % DGLA of total fatty acids (Fig. 2b) and is composed of heterologous genes encoding Δ12 desaturase of *Fusarium moniliforme* [35]; C16/18 elongase of *M. alpina* [36]; Δ9 elongase of *E. gracilis* [37] and synthetic mutant of Δ8 desaturase [38] derived from *E. gracilis*. Minimal Media + Leucine (MMLeu), High Glucose Media (HGM) and YPD medium were used as required for *Y. lipolytica* strains and cultured at 30 °C. MMLeu (per liter): 20 g of glucose; 1.7 g yeast nitrogen base without amino acids or ammonium sulfate; 0.1 g proline; 0.1 g leucine; pH 6.1. HGM (per liter): 80 g glucose, 2.58 g KH₂PO₄, 5.36 g K₂HPO₄, pH 7.5. YPD medium (per liter): 10 g of yeast extract, 20 g of Bacto peptone, and 20 g of glucose. Agar plates were prepared by addition of 20 g/l agar to liquid media.

General Techniques for Molecular Biology

Recombinant DNA techniques were used according to standard methods [39, 40]. Site-directed mutagenesis was performed according to the manufacturer's protocol (QuikChange™, Stratagene; San Diego, CA). When PCR or site-directed mutagenesis was involved in the generation of mutants and/or cloning, DNA was sequenced to verify that no additional mutations were introduced.

Total RNA was extracted from the *E. gracilis* cells using the RNA STAT-60™ reagent (Amsbio LLC., Lake forest, CA). 85 μg of mRNA was purified from 1 mg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ). Synthesis of cDNA from the *E. gracilis* mRNA was carried out using the adapter primer AP of 3'-RACE kit from Invitrogen (Carlsbad, CA) and the Smart IV oligonucleotide of BD-Clontech Creator™ Smart™ cDNA library kit (Mississauga, ON, Canada) as primers. The reverse transcription was performed with Superscript II reverse transcriptase of Invitrogen.

PCR reactions were carried out in a 50 μl total volume comprising: PCR buffer (containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris–HCl (pH 8.75), 2 mM MgSO₄, 0.1 % Triton X-100), 100 μg/mL BSA, 200 μM each deoxyribonucleotide triphosphate, 10 pmol of each primer, 10 ng cDNA of *E. gracilis* and 1 μl of Taq DNA polymerase (Epicentre Technologies, Madison, WI). The thermocycler conditions were set for 35 cycles at 95 °C for 1 min, 56 °C for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The DNA band with expected size was isolated from a 1 % agarose gel and cloned into pGEM-T easy vector (Promega, Madison, WI).

Modified 5' and 3' RACE techniques were used to obtain the full length *EgΔ5D*. The cDNA product from *E. gracilis* mRNA was used as template, and all the primers used in the 5' and 3' RACE are listed in Supplemental Table S1. Specifically, a gene specific primer ODMWP480

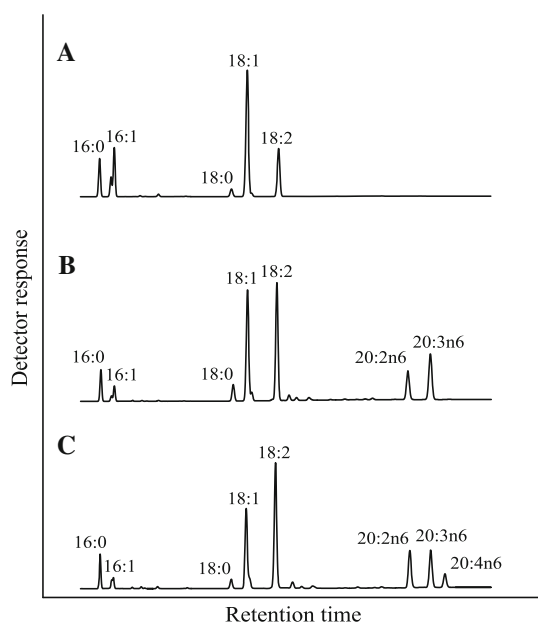


Fig. 2 Chromatograms of the fatty acid profiles. Fatty acid profile of wild type *Y. lipolytica* strain ATCC#20362 (a). Fatty acid profile of Y4036U strain, producing about 8 % HGLA (b). Fatty acid profile of Y4036U strain transformed with pDMW367-M4, producing about 3.7 % ARA of total lipids (c)

and a generic primer CDSIII 5' were used in the first round of 5' RACE. The PCR amplifications were carried out in a 50 μ l total volume, comprising: 25 μ l of *LA Taq*TM pre-mix (TaKaRa Bio Inc., Otsu, Shiga, 520-2193, Japan), 10 pmol of each primer and 1 μ l of Taq DNA polymerase (Epicentre Technologies, Madison, WI). The thermocycler conditions were the same as described above. One micro liter of this product was directly used in a second amplification, which differed from the first only in that the primers used, ODMWP479 and the generic primer DNR CDS 5' were internal to the first set of primers. As no translation initiation codon was found in the product of the first round of 5' RACE, the entire modified 5' RACE protocol was repeated using gene specific primers YL791 and YL792 as primers instead of the primers used in the first round.

A variation of a 3' RACE technique was used to isolate the C-terminal fragment of *Eg Δ 5D*. The combinations of primers ODMW469 and AUAP, and then YL470 and AUAP were used in the initial amplification and second round reaction, respectively. The PCR reactions were the same as those described for the 5' RACE.

Yarrowia Expression Vector and Transformation

Yarrowia expression vector pDMW367 contained autonomous replication sequence 18 [41] and a *URA3* gene (Genbank accession#: No. AJ306421) of *Y. lipolytica*. It also contains a *FBAIN::Eg Δ 5D:Pex20* chimeric gene. The *FBAIN* is a promoter derived from the fructose-bisphosphate aldolase gene (*FBA1*) of *Y. lipolytica* [42]. The *Eg Δ 5D* is the coding region of a wild type Δ 5 desaturase of *E. gracilis*, in which the amino acid at position 347 is arginine. The *Pex20* was a terminator sequence of *PEX20* gene (Genbank accession#: AF054613) of *Y. lipolytica*. Transformation of *Y. lipolytica* strain Y4036U was carried out as described by Chen et al. [43].

Cultivation of *Y. lipolytica* Transformants and Fatty Acid Analysis by Gas Chromatography

The *Yarrowia* expression plasmid and its derivatives were used to transform strain Y4036U individually. Transformants from each transformation were streaked onto new MMLeu plates and kept in a 30 °C incubator for 2 days. Cells from streaked plates were cultivated in 24 well blocks with 3 mL MMLeu, and incubated for 2 days at 30 °C with shaking at 200 rpm. The cells were then collected by centrifugation and resuspended in 3 mL HGM. The cells were incubated another 5 days at 30 °C with shaking at 200 rpm.

Fatty acid methyl esters from 1 ml cell culture of *Y. lipolytica* or *E. gracilis* were prepared as described [44],

except that the fatty acid methyl esters were extracted with 0.5 ml of heptane and separated by Agilent 7890A GC using hydrogen as carrier gas supplied by a hydrogen generator (Parker Hannifin, Cleveland, OH). The oven temperature was programmed from 200 to 240 °C at a rate of 25 °C/min. The proportion of each fatty acid was based on the integrated peak area of the corresponding fatty acid methyl ester as a percent relative to the sum of all integrated peaks as calculated by Agilent ChemStation Software.

Results

Isolation of Δ 5 Desaturase Gene from *E. gracilis*

Fatty acid profile analyses showed that there were moderate amounts of EDA, DGLA, ARA, EPA, docosapentaenoic acid (DPA 22:5n-3) and DHA produced in *E. gracilis* (data not shown), confirming that there was a “ Δ 9 elongase/ Δ 8 desaturase” pathway in *E. gracilis* [16, 38, 45]. The genes encoding the Δ 9 elongase, Δ 8 desaturase [16, 38], Δ 5 desaturase, Δ 17 desaturase, C20/22 elongase and Δ 4 desaturase [45] were responsible for the production of EDA, DGLA, ARA, EPA, DPA and DHA, respectively (Fig. 1).

After comparison of four Δ 5 desaturase genes, *Pi Δ 5D* from *Pythium irregulare* [46], *Pm Δ 5D* from *Phytophthora megasperma* (Genbank accession #: CAD53323), *Pt Δ 5D* from *Phaeodactylum tricorutum* [47], and *Dd Δ 5D* from *Dictyostelium discoideum* (Genbank accession #: XP_640331) as well as two Δ 8 desaturase genes, *Eg Δ 8D* from *E. gracilis* [16, 38] and *Pl Δ 8D* from *Pavlova lutheri* [48], two conserved regions, GHH(I/V)YTN and N(Y/F)Q(V/D)EHH (Fig. 3) were selected to design primers to amplify a portion of *Eg Δ 5D*. To reduce the degeneracy of the primers, four primers (Supplemental Table S1: 5-1A to 1D) were generated for conserved region 1 and four primers (Supplemental Table S1: 5-5AR to 5-5DR) for the anti-sense strand of conserved region 2. One DNA fragment amplified with primers 5-1B and 5-5DR was cloned into pGEM-T Easy vector to generate pT-F10-1. DNA sequence showed that a 590 bp insert of pT-F10-1 encoded an amino acid sequence with 38 % identity and 53 % similarity to the amino acid sequence of the Δ 8-sphingolipid desaturase of *Thalassiosira pseudonana* (*Ts Δ 8D*, Genbank accession #: AAX14502), and 37 % identity and 52 % similarity with *Pt Δ 5D* [47]. These data suggested that the 590 bp DNA fragment might be a part of a desaturase gene of *E. gracilis*. This gene was designated as putative *Eg Δ 5D*.

5' and 3' RACE techniques were used to extend the 590 bp region of the putative *Eg Δ 5D* (Fig. 4). A 797 bp

A

	Position	Conserved Region 1	Position
PtΔ5D	208	QHWT-HHAYTNHAE MDPD	222
PmΔ5D	228	QHVVGH HHIYTNVAGADPD	245
PiΔ5D	207	QHVVGH HHIYTNVAGADPD	224
DdΔ5D	210	QHVI GHHLYTNV RNADPD	227
EgΔ8D	182	RHNA-HHSA TNVQGHDPD	198
PvΔ8D	208	RHNT-HHVC TNE DGS DDPD	224

B

	Position	Conserved Region 2	Position
PtΔ5D	292	TGGLNFQVEHHLFPRMSS	409
PmΔ5D	407	TGALNYQVTHHLFPGV SQ	424
PiΔ5D	386	CGALNYQVTHHLYPG I SQ	403
DdΔ5D	394	SGGLNLQVTHHCFPTIAQ	411
EgΔ8D	352	FGGLNYQIEHHLWPTLPR	369
PvΔ8D	362	TGFISLQTEHHLFPMMP T	379

Fig. 3 Alignment of the conserved regions among some Δ5 and Δ8 desaturases. The amino acid sequence alignment was performed with Clustal W analysis (MegAlign™ program of DNASTAR software). Identical residues are shaded in black. PtΔ5D, Δ5 desaturase from *P. tricorutum* (29, GenBank accession #: AAL92562); PmΔ5D, Δ5 desaturase from *P. megasperma* (GenBank accession #: CAD53323); PiΔ5D, Δ5 desaturase from *P. irregulare* (30, GenBank accession #: AAL13311); DdΔ5D, Δ5 desaturase from *D. discoideum* (GenBank accession #: XP_640311); EgΔ8D, Δ8 desaturase from *E. gracilis* (24, GenBank accession #: AAD45877) and PvΔ8D, Δ8 desaturase from *P. lutheri* [48]

DNA fragment with no translation initiation codon was isolated by the first round of 5' RACE. This 797 bp DNA fragment had a 238 bp overlap with the 5' end of the 590 bp fragment of pT-F10-1 and provided 559 bp of 5' upstream sequence. A 273 bp DNA fragment was generated by the second round 5' RACE experiment. This 273 bp DNA fragment had 253 bp overlap with the 5' part of the 797 bp DNA fragment and provided 20 bp of 5' upstream sequence. Seventeen [17] bp of the 20 bp encoded the N-terminal portion of the putative *EgΔ5D*, including the translation initiation codon. A 464 bp DNA sequence was identified by one round of 3' RACE. The first 184 bp of the 464 bp fragment encoded the C-terminal coding region, including the translation stop codon, of the putative *EgΔ5D*.

Assembly of the 5' region, the original 590 bp fragment and the 3' region resulted in a 1,633 bp contig, comprising the complete coding region with additional untranslated 5' and 3' ends (Fig. 4). The coding region of the putative *EgΔ5D* is 1,350 bp in length and encodes a peptide of 449 amino acids. BlastP searches using the full length putative *EgΔ5D* as the query sequence showed that it shares 39 % identity and 56 % similarity with PtΔ5D [47]; 37 % identity and 55 % similarity with TsΔ8D (Genbank accession #: AAX14502). Amino acid sequence alignment performed with the Clustal W analysis (MegAlign™

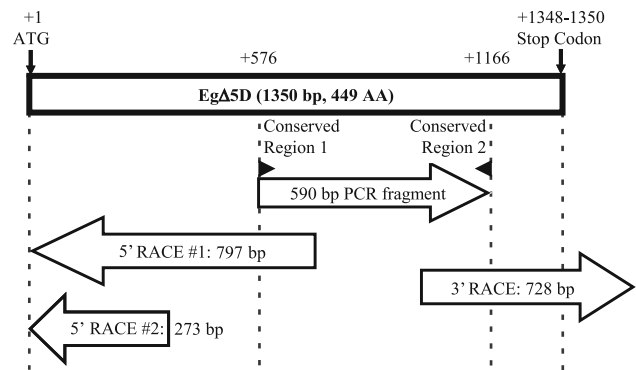


Fig. 4 Graphical representation of the assembly of full length *EgΔ5D*. A 590 bp DNA fragment encoding a portion of *EgΔ5D* was isolated by PCR amplification with degenerate primers. The N-terminal part was isolated by two round of 5' RACE, and the C-terminal portion was isolated by one round of 3' RACE

program of DNASTAR software) showed that *EgΔ5D* has <30 % identity with some represent Δ5 desaturases such as *IgΔ5D* from *I. galbana* (AEA72469); *MaΔ5D* from *M. alpina* [24, 25, 34]; *PiΔ5D* [46]; *OtΔ5D* from *O. tauri* (Genbank accession #: XP_003082424) and *TaΔ5D* from *T. aureum* [49]. Further analyses showed that *EgΔ5D* has only 20 % and 25.5 % identity with *EgΔ8D* [16] and *EgΔ4D* [45] desaturases of *E. gracilis*, respectively. It was found that the PCR products for the full length coding region of putative *EgΔ5D* had two versions, both having identical nucleotide sequence except at base pair positions 1,039 and 1,041. This disagreement resulted in a codon change from CGA to AGC. As such, one PCR product indicated arginine at position 347, whereas the second indicated serine. It was hypothesized that this discrepancy was raised at the stage of PCR amplification or during cDNA generation.

Determination of Δ5 Desaturase Activity and Topology Model of *EgΔ5D*

To study the function of the putative *EgΔ5D*, plasmid pDMW367 was generated to express the *EgΔ5D* coding region under the control of the strong constitutive FBAIN promoter [42] from *Y. lipolytica*. The four restriction sites (i.e., *Bgl*II, *Eco*RI, *Hind*III and *Nco*I) inside the coding region of *EgΔ5D* in pDMW367 were removed by site-directed mutagenesis to generate pDMW367-M4 (Supplemental Fig. S1). The amino acid sequence of *EgΔ5D* is identical in pDMW367 and pDMW367-M4 constructs, and the amino acid at position 347 is an arginine.

The pDMW367 and pDMW367-M4 constructs were used to transform DGLA producing strain Y4036U. There was no ARA produced in the parent strain Y4036U (Fig. 2b), while there was about 3.7 % ARA and 11.5 % DGLA produced in transformants of Y4036U with either

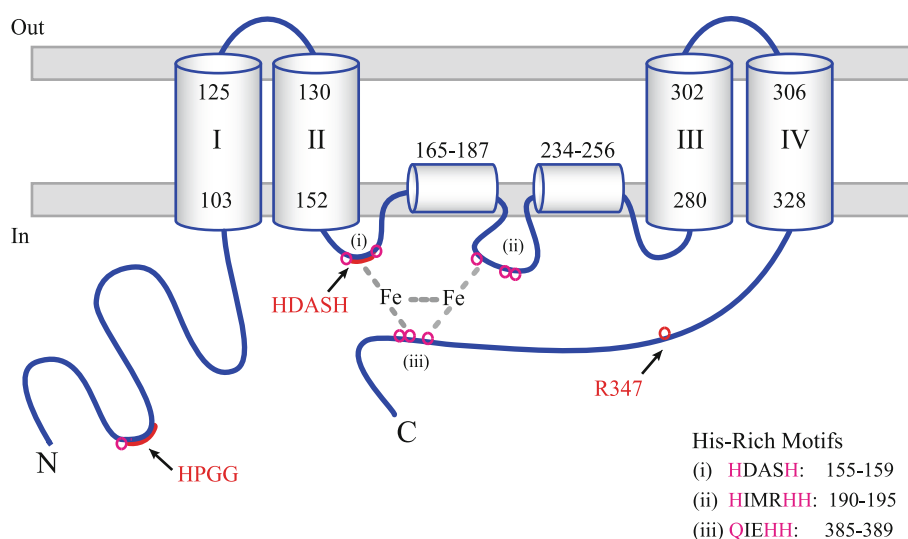


Fig. 5 Topology model of *EgΔ5D*. *EgΔ5D* is a membrane diiron protein with three His-rich motifs. The $HX_{(3,4)}H$ motif, HDASH, is located from residues 155 to 159; the $HX_{(2,3)}HH$ motif, HIMRHH, located from residues 190 to 195, and the $(H/Q)X_{(2,3)}HH$ motif,

QIEHH, located from residues 385 to 389. *EgΔ5D* has four transmembrane domains (I–IV) and two hydrophobic stretches (residues from 165 to 187, and 234 to 256). Its N-terminal cytochrome b_5 domain and C-terminal region are located in the cytoplasm

the pDMW367 or pDMW367-M4 construct (Fig. 2c). These results demonstrated that *EgΔ5D* indeed encodes a $\Delta 5$ desaturase. *EgΔ5D* could convert DGLA to ARA with a conversion of about 24.3 %. The conversion of DGLA to ARA was calculated according to the formula: $([ARA \text{ product}]/[DGLA \text{ substrate} + ARA \text{ product}])$. The pDMW367 and pDMW367-M4 constructs were also used to transform strain Y2224, a FOA resistant mutant of wild type strain ATCC#20362. There was no GLA produced in the transformants with either the pDMW367 or pDMW367-M4 construct (data not shown). These results demonstrated that *EgΔ5D* does not have $\Delta 6$ desaturase activity; it is not a bifunctional enzyme.

Like other fatty acid desaturases, *EgΔ5D* is also a membrane-bound enzyme and belongs to a super-family of membrane di-iron proteins with three His-rich motifs: $HX_{(3,4)}H$, $HX_{(2,3)}HH$ and $(H/Q)X_{(2,3)}HH$. These His residues have been predicted to be located in the cytoplasmic face of the membrane and have been shown to be very important for enzyme activity [33]. Within *EgΔ5D*, these 3 His-rich motifs are the HDASH motif located from residue 155 to 159, the HIMRHH motif located from residue 190 to 195, and the QIEHH motif located from residue 385 to 389. The third His-rich motif contains a glutamine substitution that is common to other front-end desaturases. Based on transmembrane domain analysis (TMHMM Server v. 2.0, Center for Biological Sequence Analysis, BioCentrum-DTU, Technical University of Denmark, DK-2800 Lyngby, Denmark) and the location of the His-rich motifs, a topology model of *EgΔ5D* was developed (Fig. 5). The model shows that the N-terminal cytochrome b_5 domain is located in the

cytosol. The topology model also predicts that *EgΔ5D* has a total of four transmembrane regions (amino acid residues 103–125, 130–152, 280–302 and 306–328) and two hydrophobic regions (amino acid residues 165–187, and 234–256). These hydrophobic segments are not membrane-spanning, and may represent hydrophobic patches located closed to the di-iron active site. Because the substrates for the desaturase is highly hydrophobic, they will likely partition into the lipid bilayer. Therefore we purport that the di-iron active site assembled from these His-clusters may occur at or very near the membrane surface.

The HPGG Motif is Important, but not Necessary for $\Delta 5$ Desaturase Activity of *EgΔ5D*

It has been suggested that the highly conserved HPGG motif plays a crucial role in heme group assembly, protein folding and stabilization in cytochrome b_5 proteins, with the histidine residue functioning as an axial heme ligand where a peptide chain reversal occurs [50]. Previous studies have demonstrated that the heme-binding HPGG motif, and in particular, the histidine residue, is essential for enzyme activity of desaturases with cytochrome b_5 domain [51–53]. Although sequence divergence in the vicinity of the HPGG motif is normal, the HPGG motif itself has been conserved throughout the evolution of all the $\Delta 5$ desaturase genes [54]. Thus it was claimed to be a characteristic of $\Delta 5$ desaturases and necessary for its function to convert DGLA to ARA [34].

To assess the functional significance of the HPGG motif (position 33–36) of *EgΔ5D*, we first elected the proline

Table 1 $\Delta 5$ Desaturase activity of *EgΔ5D* with HxGG mutations

Gene name	Sequence of HPGG motif	$\Delta 5$ Conversion (%)	% of wild type <i>EgΔ5D</i>
<i>EgΔ5D</i>	HPGG	24.2	100
<i>EgΔ5D-34A</i>	HaGG	22.7	93.5
<i>EgΔ5D-34C</i>	HcGG	22.6	93.2
<i>EgΔ5D-34D</i>	HdGG	12.5	51.6
<i>EgΔ5D-34E</i>	HeGG	14.7	60.5
<i>EgΔ5D-34F</i>	HfGG	17.9	73.9
<i>EgΔ5D-34G</i>	HgGG	23.8	98.2
<i>EgΔ5D-34H</i>	HhGG	21.3	87.8
<i>EgΔ5D-34I</i>	HiGG	18.3	75.4
<i>EgΔ5D-34K</i>	HkGG	22.7	93.8
<i>EgΔ5D-34L</i>	HlGG	17.0	70.0
<i>EgΔ5D-34M</i>	HmGG	19.0	78.4
<i>EgΔ5D-34N</i>	HnGG	19.8	81.5
<i>EgΔ5D-34Q</i>	HqGG	19.8	81.7
<i>EgΔ5D-34R</i>	HrGG	19.4	79.9
<i>EgΔ5D-34S</i>	HsGG	20.4	84.1
<i>EgΔ5D-34T</i>	HtGG	19.6	80.7
<i>EgΔ5D-34V</i>	HvGG	20.2	83.4
<i>EgΔ5D-34W</i>	HwGG	22.2	91.7
<i>EgΔ5D-34Y</i>	HyGG	17.7	73.1

Average of 6 samples for each construct containing different mutations

residue at position 34 (P34) as a target for amino acid substitution. Single amino acid mutations were carried out to generate all 19 amino acid substitution mutants (HxGG). Table 1 shows that the P34 residue could be substituted with several different amino acids without significantly impacting the $\Delta 5$ desaturase activity of *EgΔ5D*. The *EgΔ5D-34A*, *EgΔ5D-34C*, *EgΔ5D-34K* or *EgΔ5D-34W* mutants exhibited >90 % of the wild type *EgΔ5D* activity. The *EgΔ5D-34G* mutant was functionally equivalent to the wild type *EgΔ5D*.

Next, we studied the significance of the second glycine residue at position 36 (G36) within the HPGG motif of *EgΔ5D* using the same approach as that for P34. Table 2 shows that the G36 residue within the HPGG motif could be substituted with several different amino acids without significantly impacting the $\Delta 5$ desaturase activity of *EgΔ5D*. The *EgΔ5D-36S* or *EgΔ5D-36D* mutant had about 100.8 or 99.2 % of $\Delta 5$ desaturase activity when compared to *EgΔ5D*, respectively.

The above functional studies at the P34 and G36 positions within the HPGG motif of *EgΔ5D* demonstrated that the HPGG motif could be changed without impacting the $\Delta 5$ desaturase activity. Specifically, the *EgΔ5D-34G*, *EgΔ5D-36S* and *EgΔ5D-36D* mutants were functionally equivalent to the wild type *EgΔ5D*. Comparison of the

Table 2 $\Delta 5$ Desaturase activity of *EgΔ5D* with HPGx mutations

Gene name	Sequence of HPGG motif	$\Delta 5$ Conversion (%)	% of wild type <i>EgΔ5D</i>
<i>EgΔ5D</i>	HPGG	24.2	100
<i>EgΔ5D-36A</i>	HPGa	18.3	75.6
<i>EgΔ5D-36C</i>	HPGc	6.5	26.8
<i>EgΔ5D-36D</i>	HPGd	24.0	99.2
<i>EgΔ5D-36E</i>	HPGe	21.1	86.9
<i>EgΔ5D-36F</i>	HPGf	3.3	13.4
<i>EgΔ5D-36H</i>	HPGh	18.1	74.8
<i>EgΔ5D-36I</i>	HPGi	1.5	6.4
<i>EgΔ5D-36K</i>	HPGk	19.0	78.3
<i>EgΔ5D-36L</i>	HPGl	9.1	37.7
<i>EgΔ5D-36M</i>	HPGm	13.4	55.2
<i>EgΔ5D-36N</i>	HPGn	19.5	80.3
<i>EgΔ5D-36P</i>	HPGp	18.0	74.1
<i>EgΔ5D-36Q</i>	HPGq	19.9	82.1
<i>EgΔ5D-36R</i>	HPGr	15.5	63.8
<i>EgΔ5D-36S</i>	HPGs	24.4	100.8
<i>EgΔ5D-36T</i>	HPGt	22.8	93.9
<i>EgΔ5D-36V</i>	HPGv	1.9	8.0
<i>EgΔ5D-36W</i>	HPGw	15.1	62.2
<i>EgΔ5D-36Y</i>	HPGy	11.2	46.3

Average of 6 samples for each construct containing different mutations

ARA in unesterified fatty acid (FFA), phospholipid (PL) and neutral lipid (NL) pools of *Yarrowia* transformants with *EgΔ5D* and *EgΔ5D-34G* showed that the P34G mutation did not affect the ARA distribution in these pools (Supplemental Fig. S2).

Improvement of $\Delta 5$ Desaturase Activity of *EgΔ5D* by Amino Acid Substitution Within the HDASH Motif

The HDASH motif was also claimed as one of the characteristics of $\Delta 5$ desaturases and necessary for its function to convert DGLA to ARA [34]. To test the hypothesis that the exact sequence of the HDASH motif (position 155–159) of *EgΔ5D* was required, we first selected the alanine residue at position 157 (A157) as a target. The $\Delta 5$ desaturase activity attributed to each mutation at A157 is summarized in Table 3. The data showed that almost all mutations at A157 greatly reduced the $\Delta 5$ desaturase activity of *EgΔ5D*. However, the *EgΔ5D-157G* and *EgΔ5D-157S* mutants retained about 96 and 94 % activity of wild type *EgΔ5D*, respectively.

We also studied the significance of the serine residue at position 158 (S158) within the HDASH motif of *EgΔ5D*. Table 4 shows that the S158 could be substituted with either an alanine or a glycine without substantially

Table 3 $\Delta 5$ Desaturase activity of *EgΔ5D* with HDxSH mutations

Gene name	Sequence of HDASH motif	$\Delta 5$ Conversion (%)	% of wild type <i>EgΔ5D</i>
<i>EgΔ5D</i>	HDASH	24.8	100
<i>EgΔ5D-157C</i>	HDcSH	10.7	43.1
<i>EgΔ5D-157D</i>	HDdSH	1.0	4.0
<i>EgΔ5D-157E</i>	HDeSH	0.9	3.6
<i>EgΔ5D-157F</i>	HDfSH	1.0	4.0
<i>EgΔ5D-157G</i>	HDgSH	23.8	96
<i>EgΔ5D-157H</i>	HDhSH	1.0	4.0
<i>EgΔ5D-157I</i>	HDiSH	0.9	3.6
<i>EgΔ5D-157K</i>	HDkSH	1.0	4.0
<i>EgΔ5D-157L</i>	HDlSH	1.1	4.4
<i>EgΔ5D-157M</i>	HDmSH	1.0	4.0
<i>EgΔ5D-157N</i>	HDnSH	1.1	4.4
<i>EgΔ5D-157P</i>	HDpSH	2.3	9.3
<i>EgΔ5D-157Q</i>	HDqSH	0.6	2.4
<i>EgΔ5D-157R</i>	HDrSH	0.8	3.2
<i>EgΔ5D-157S</i>	HDsSH	23.3	94
<i>EgΔ5D-157T</i>	HDtSH	1.0	4.0
<i>EgΔ5D-157V</i>	HDvSH	0.3	1.2
<i>EgΔ5D-157W</i>	HDwSH	0.9	3.6
<i>EgΔ5D-157Y</i>	HDySH	0.7	2.8

Average of 3 samples for each construct containing different mutations

impacting the $\Delta 5$ desaturase activity of *EgΔ5D*. Specifically, the *EgΔ5D-158A* mutant had about 100.9 % activity of wild type *EgΔ5D*, while the *EgΔ5D-158G* mutation had about 107.7 % activity of wild type *EgΔ5D*. These data demonstrated that the HDASH motif could be changed without significantly reducing the $\Delta 5$ desaturase activity. In fact, the enzyme activity of *EgΔ5D* could be improved by substitution of the S158 within the HDASH motif with a glycine residue.

Both HPGG and HDASH Motifs were not Necessary in the Exact Form as Encoded for $\Delta 5$ Desaturase Activity of *EgΔ5D*

In order to study whether *EgΔ5D* could maintain strong $\Delta 5$ desaturase activity with mutations in both HPGG and HDASH motifs, we generated a series of double mutants. The results (Table 5) demonstrate that $\Delta 5$ desaturases could be constructed having variant HPGG and HDASH motifs that retain at least 64 % of $\Delta 5$ desaturase activity when compared to the wild type.

The proline residue within the HPGG motif can be substituted with glycine with simultaneous substitution of either (1) the alanine residue within the HDASH motif for glycine or (2) the serine residue within the HDASH motif

Table 4 $\Delta 5$ Desaturase activity of *EgΔ5D* with HDxH mutations

Gene name	Sequence of HDASH motif	$\Delta 5$ Conversion (%)	% of wild type <i>EgΔ5D</i>
<i>EgΔ5D</i>	HDASH	23.3	100
<i>EgΔ5D-158A</i>	HDAaH	23.5	100.9
<i>EgΔ5D-158C</i>	HDAcH	17.9	76.8
<i>EgΔ5D-158D</i>	HDA dH	2.8	12.0
<i>EgΔ5D-158E</i>	HDAeH	1.9	8.2
<i>EgΔ5D-158F</i>	HDAfH	1	4.3
<i>EgΔ5D-158G</i>	HDAgH	25.1	107.7
<i>EgΔ5D-158H</i>	HDAhH	1.6	6.9
<i>EgΔ5D-158I</i>	HDaiH	1.1	4.7
<i>EgΔ5D-158K</i>	HDAkH	1	4.3
<i>EgΔ5D-158L</i>	HDAlH	1.1	4.7
<i>EgΔ5D-158M</i>	HDAmH	2.3	9.9
<i>EgΔ5D-158N</i>	HDA nH	16.5	70.8
<i>EgΔ5D-158P</i>	HDApH	1.2	5.2
<i>EgΔ5D-158Q</i>	HDAqH	10.4	44.6
<i>EgΔ5D-158R</i>	HDArH	10.0	42.9
<i>EgΔ5D-158T</i>	HDA tH	9.6	41.2
<i>EgΔ5D-158V</i>	HDAvH	1.5	6.4
<i>EgΔ5D-158W</i>	HDAwH	9.3	40.0
<i>EgΔ5D-158Y</i>	HDAyH	1.1	4.7

Average of 3 samples for each construct containing different mutations

for alanine or glycine. The proline residue within the HPGG motif can also be substituted with histidine with simultaneous substitution of the serine residue within the HDASH motif for either alanine or glycine. And, the second glycine residue within the HPGG motif can be substituted with serine with simultaneous substitution of the serine residue within the HDASH motif for either alanine or glycine. Specifically, the *EgΔ5D-34G/157G*, *EgΔ5D-34G/158A* and *EgΔ5D-34H/158G* double mutants had more than 80 % of the $\Delta 5$ desaturase activity of *EgΔ5D*, while *EgΔ5D-34G/158G* had about 97 % $\Delta 5$ desaturase activity of *EgΔ5D*. Further analyses showed that the ARA distribution in FFA, PL and NL pools of *Yarrowia* transformants with *EgΔ5D-34G/158G* was similar to *Yarrowia* transformants with *EgΔ5D-34G* or *EgΔ5D* (Supplemental Fig. S2), suggesting that the simultaneous substitutions (P34G and S158G) within HPGG and HDASH motifs did not change desaturase substrate specificity.

Increased Substrate Conversion of *EgΔ5D-34G/158G* with Double Mutations in HPGG and HDASH Motifs

In order to increase the substrate conversion of *EgΔ5D-34G/158G*, we optimized the codon usage of the

Table 5 $\Delta 5$ Desaturase activity of *Eg $\Delta 5D$* with mutants simultaneously comprising mutations within HPGG and HDASH motifs

Gene name	Sequence of HPGG motif	Sequence of HDASH motif	$\Delta 5$ Conversion (%)	% of wild type <i>Eg$\Delta 5D$</i>
<i>Eg$\Delta 5D$</i>	HPGG	HDASH	27.5	100
<i>Eg$\Delta 5D$-34G157G</i>	HgGG	HDgSH	22.9	83
<i>Eg$\Delta 5D$-34G158A</i>	HgGG	HDAaH	24.3	88
<i>Eg$\Delta 5D$-34G158G</i>	HgGG	HDAgH	26.8	97
<i>Eg$\Delta 5D$-34H158A</i>	HhGG	HDAaH	18.7	68
<i>Eg$\Delta 5D$-34H158A</i>	HhGG	HDAgH	22	80
<i>Eg$\Delta 5D$-36S158A</i>	HPGs	HDAaH	17.5	64
<i>Eg$\Delta 5D$-36S158G</i>	HPGs	HDAgH	18.9	69

Average of 6 samples for each construct containing different mutations

N-terminal portion of the gene for expression in *Y. lipolytica*. The codon-optimized *Eg $\Delta 5D$ -34G/158G*, designated as “*Eg $\Delta 5M$* ”, had 48 bp changed in the first 204 bp of the coding region (23.5 %; Fig. 6), which resulted in optimization of 43 codons of the first 68 amino acids within the N-terminus of the protein (63.2 %). The amino acid sequence encoded by the codon-optimized *Eg $\Delta 5M$* was identical to that of the *Eg $\Delta 5D$ -34G/158G*. *Eg $\Delta 5M$* was used to replace the *Eg $\Delta 5D$* of pDMW367-M4 to generate pDMW367-5M, containing a *FBAIN::Eg $\Delta 5M$::Pex20* chimeric gene.

We then studied the importance of the arginine or serine at position 347 that were found in the original clones of *Eg $\Delta 5D$* . Based on *Eg $\Delta 5M$* , the CGA codon for arginine at position 347 was changed to AGC codon to encode for serine, which was designated as *Eg $\Delta 5M1$* . The synthetic *Eg $\Delta 5M1$* was used to replace the *Eg $\Delta 5D$* of pDMW367-M4 to generate pDMW367-5M1, containing an *FBAIN::Eg $\Delta 5M1$::Pex20* chimeric gene.

The $\Delta 5$ desaturase activity of *Eg $\Delta 5D$* , *Eg $\Delta 5M$* and *Eg $\Delta 5M1$* is summarized in Table 6. GC analyses determined that there were about 3.6 % ARA and 10.8 % DGLA, 4.0 % ARA and 11.2 % DGLA, and 4.1 % ARA and 10.8 % DGLA of total fatty acids produced in the *Yarrowia* transformants with pDMW367-M4, pDMW367-5M, and pDMW367-5M1, respectively. It showed that the wild-type *Eg $\Delta 5D$* converted about 24.8 % of DGLA to ARA; *Eg $\Delta 5M$* converted 26.7 % of DGLA to ARA; and, *Eg $\Delta 5M1$* converted 27.6 % of DGLA to ARA. The fatty acid profile of *Yarrowia* transformants with *Eg $\Delta 5M1$* was

<i>Eg$\Delta 5D$-34G158G</i>	ATGGCTCTCAGTCTTACCACAGAACAGCTG	30
<i>Eg$\Delta 5M$</i>	ATGGCCCTGTCTCTCACTACCAGAACAGCTC	30
<i>Eg$\Delta 5D$-34G158G</i>	TTAGAACGCCCTTGATTTGGTTGCGGATTTGAT	60
<i>Eg$\Delta 5M$</i>	CTGGAGCGACCTTGATCTCGTTCGCTATTCGAT	60
<i>Eg$\Delta 5D$-34G158G</i>	GGCATCCTCTACGACCTTGAGGGCTTGCC	90
<i>Eg$\Delta 5M$</i>	GGTATCCTGTACGACCTCGAGGGCCTTGCC	90
<i>Eg$\Delta 5D$-34G158G</i>	AAAAGTTCATGGTGGAGGAGATTTGATTCTC	120
<i>Eg$\Delta 5M$</i>	AAGGTGCATGGTGGTGGAGACCTCATTCTG	120
<i>Eg$\Delta 5D$-34G158G</i>	GCTTCTGGTGCCTTGATGCCTCCCTCTC	150
<i>Eg$\Delta 5M$</i>	GCCTCTGGAGCCTCGACGCCTCTCCCTC	150
<i>Eg$\Delta 5D$-34G158G</i>	TTTTATTCAATGCATCCATACGTCAAACCG	180
<i>Eg$\Delta 5M$</i>	TTCTACTCTATGCATCCCTACGTCAAAGCC	180
<i>Eg$\Delta 5D$-34G158G</i>	GAGAACTCCAAATTGCTTCACACAG	204
<i>Eg$\Delta 5M$</i>	GAGAACTCCAAGCTCTGCACCAA	204

Fig. 6 Comparison of the 5' portion (204 bp) of *Eg $\Delta 5D$ -34G158G* and its codon optimized version, *Eg $\Delta 5M$* . The DNA sequence alignment was performed with Clustal W analysis (MegAlign™ program of DNASTAR software). Identical residues are shaded in black

almost identical to the profile of *Yarrowia* transformants with pDMW367-M4 as shown in Fig. 2c, except that more ARA was produced. These data demonstrated that the codon optimization of *Eg $\Delta 5D$* improved its substrate conversion efficiency. Further, the amino acid at position 347 did affect the $\Delta 5$ desaturase activity of *Eg $\Delta 5D$* , with a serine residue preferred over an arginine residue.

Discussion

Y. lipolytica has an established history of robust fermentation performance at commercial scale for processes including the production of food-grade citric acid for human consumption and single-cell protein for animal feeds [55]. Recently, *Y. lipolytica* has been used as a host for production of lipid-based compounds [14, 56, 57]. Some *Y. lipolytica* strains are oleaginous organisms that are able to accumulate up to 40 % dry cell weight as oil when starved for nitrogen in the presence of excess glucose as carbon source. However, LNA is the only PUFA that *Y. lipolytica* can synthesize de novo (Fig. 2a). Therefore, it

Table 6 $\Delta 5$ Desaturase activity of *Eg $\Delta 5D$* and codon optimized *Eg $\Delta 5M$* and *Eg $\Delta 5M1$*

Gene name	Sequence of HPGG motif	Sequence of HDASH motif	Sequence at residue 347	$\Delta 5$ Conversion (%)
<i>Eg$\Delta 5D$</i>	HPGG	HDASH	R	24.8
<i>Eg$\Delta 5M$</i>	HgGG	HDAgH	R	26.5
<i>Eg$\Delta 5M1$</i>	HgGG	HDAgH	S	27.6

Average of 6 samples for each construct containing different mutations

is necessary to isolate genes encoding enzymes for every step of the “desaturation and elongation” pathways (Fig. 1) before genetically engineering *Y. lipolytica* to produce ARA, EPA and DHA oil. $\Delta 5$ desaturase is the enzyme responsible for the conversion of DGLA to ARA, and ETA to EPA. Although several $\Delta 5$ desaturase genes have been isolated from various organisms [26], more effective enzymes may help to improve the production of commercially important LC-PUFA.

Previous studies have indicated that *Euglena* was able to synthesize ARA, EPA and DHA through the “ $\Delta 9$ elongase/ $\Delta 8$ desaturase” pathway [16, 38, 45]. In this report, the gene encoding a $\Delta 5$ desaturase from *E. gracilis* was isolated and characterized. Our results indicated two nucleotide sequences with a difference of two base pairs that would result in either arginine or serine at position 347. This discrepancy was most likely generated from PCR amplification or during cDNA generation. BlastP searches showed that the amino acid sequence of *Eg $\Delta 5D$* shares <40 % identity with any $\Delta 5$ desaturase found in Genbank; *Pt $\Delta 5D$* [47] was the most similar one (about 39 %), suggesting that the primary structure of *Eg $\Delta 5D$* is quite different from those $\Delta 5$ desaturase genes previously isolated. Amino acid sequence alignment also shows that *Eg $\Delta 5D$* has about 20 % identity with *Eg $\Delta 8D$* [16, 38] and about 25.5 % identity with *Eg $\Delta 4D$* [45]. These data suggest that *Eg $\Delta 5D$* is evolutionary closer to $\Delta 5$ desaturase than the $\Delta 4$ or $\Delta 8$ desaturases. Functional analyses of *Eg $\Delta 5D$* in *Y. lipolytica* strains Y4036U and Y2224 revealed that it has strong $\Delta 5$ desaturase activity, with more than 24 % substrate conversion of DGLA to ARA, and it is not a $\Delta 5/\Delta 6$ bifunctional enzyme.

The HPGG motif is expected to be on the cytochrome b_5 surface, in contact with the heme through van der Waals interactions [27]. The conserved HPGG motif was thought to be essential in maintaining cytochrome b_5 electron transfer function, with the histidine serving as a heme axial ligand [50]. Substitution of the histidine residue of the HPGG motif with alanine in the $\Delta 6$ desaturase cytochrome b_5 domain of starflower, rat and algae abolished $\Delta 6$ desaturase activity [51–53]. It is expected that H33 of *Eg $\Delta 5D$* should also be essential for its function.

The HPGG motif itself has a unique structure. The proline residue of the HPGG stretch is located in a turn between two consecutive helices (Fig. 5) and was thought to be important in protein folding and in maintaining cytochrome b_5 protein stability [32]. The three-carbon side chain of proline is bonded to both the nitrogen and the carbon of the peptide backbone to form a five-member ring that greatly restricted its conformational freedom. The nonpolar characteristic of this ring structure may create a hydrophobic spot within the HPGG motif. On the other hand, the glycine possesses the smallest amino acid side

chain, hydrogen, which can allow for greater flexibility in local structure. It is likely that the combination of proline and glycine residues within the cytochrome b_5 HPGG motif is an important factor affecting both the structural position of the hydrophobic heme pocket and the appropriate orientation of the heme group within the heme pocket relative to the desaturase catalytic site. Surprisingly, our results indicate that the proline residue of the HPGG motif is not essential for electron transfer from the heme group of the cytochrome b_5 domain to the catalytic diiron cluster of *Eg $\Delta 5D$* . Most substitution mutants at P34 displayed at least 70 % of the wild type *Eg $\Delta 5D$* activity. The *Eg $\Delta 5D$ -34G* (HgGG) mutant had greater than 98 % of the wild type *Eg $\Delta 5D$* activity, demonstrating that the proline residue of HPGG motif is not required for the enzyme activity of *Eg $\Delta 5D$* (Table 1).

It is noteworthy that aspartate substitutions in *Eg $\Delta 5D$ -34D* and *Eg $\Delta 5D$ -36D* exhibit different effects on desaturase activity. Compared to free cytochrome b_5 , while there are several conserved acidic amino acids, there is a characteristic reduction in the number of aspartate and glutamate residues in the vicinity of the HPGG motif of cytochrome b_5 domain of desaturases. This reduced number of acidic residues around the heme pocket is thought to contribute to stabilizing nonpolar intermolecular interactions between the cytochrome b_5 and desaturase domains [27, 54]. Substitutions involving aspartate or glutamate residues in the HPGG motif may affect the interface geometry of electron donor/acceptor docking that is exhibited in desaturase activity due to altered electron transfer. We also found that substitutions for G36 of *Eg $\Delta 5D$* resulted in mutants with strong $\Delta 5$ desaturase activity. The most functional mutants were the small, slightly polar serine replacement, *Eg $\Delta 5D$ -36S*, and the acidic substitution with aspartate, *Eg $\Delta 5D$ -36D*. The activities of these two mutants are about the same as the wild type *Eg $\Delta 5D$* (Table 2).

The amino acid sequence of the first His-rich motif, HX_(3, 4)H, of *Eg $\Delta 5D$* is HDASH located from residues 155 to 159. The HDASH motif has been suggested as one of the characteristics of $\Delta 5$ desaturases and necessary for its function to convert DGLA to ARA in any transformed organisms [34]. Sequence analyses showed that there are natural variants of the HDASH motif in $\Delta 5$ desaturases, for example, *Pt $\Delta 5D$* [46] has the sequence of HDsSH, the $\Delta 5$ desaturase from *Thraustochytrium sp.* ATCC 21685 (GenBank accession #: AAM09687) has the sequence of HemgH, the $\Delta 5$ desaturase from *Leishmania major strain Friedlin* (GenBank accession #: CAJ07076) has the sequence of HeAgH, the $\Delta 5$ desaturase from Atlantic salmon (GenBank accession #: AAL82631) has the sequence of HDyGH, and *Pt $\Delta 5D$* [47] has the sequence of HDAnH in the corresponding location. This suggests that the HDASH

motif is not an invariant characteristic of $\Delta 5$ desaturases, and may be not required for $\Delta 5$ desaturase activity. We suggest that the two His residues of HDASH motif participate in the coordination of the diiron center (Fig. 5), but the other three residues (DAS) residues between the two His residues can be modified.

Systemic substitution studies (Tables 3, 4) at positions A157 and S158 within the HDASH motif of *EgΔ5D* demonstrated that these two residues could be replaced, and the mutants retained good $\Delta 5$ desaturase activity. The *EgΔ5D-157G* and *EgΔ5D-157S* mutants had about 96 and 94 % of the wild type *EgΔ5D* activity, respectively. Since *PiΔ5D* has an HDsSH motif [46], it is not surprising that *EgΔ5D-157S* with sequence HDsSH functioned well in *Yarrowia*. We also found that S158 could be substituted with either alanine or glycine. The alanine, glycine and serine are interchangeable within the HDASH motif of *EgΔ5S*; furthermore, the enzyme activity of *EgΔ5D* could be improved with a motif of HDAGH instead of HDASH (Table 4).

To determine whether at least one motif, HPGG or HDASH, is required for the enzyme activity of *EgΔ5D*, a series of mutants with mutations in both the HPGG and HDASH motifs was generated (Table 5). Some double mutants such as *EgΔ5D-34G/157G*, *EgΔ5D-34G/158A* and *EgΔ5D-34H/158G* had more than 80 % of wild type *EgΔ5D* activity, while *EgΔ5D-34G/158G* had almost the same activity as wild type *EgΔ5D*. Therefore, neither the HPGG nor the HDASH motif is necessary in the exact form as encoded for the activity of *EgΔ5D*.

Distribution analyses (Supplemental Fig. S2) of ARA in FFA, PL and NL pools of *Yarrowia* transformants with *EgΔ5D* shows that more ARA loaded in PL pool than that in FFA pool, suggesting that *EgΔ5D* is also an acyl-lipid desaturase, just like other front-end desaturases from lower plants, fungi and algae [26]. ARA distribution comparison of *Yarrowia* transformants with *EgΔ5D-34G* and *EgΔ5D-34G/158G* with wild type *EgΔ5D* shows that either single mutation within HPGG motif (P34G), or simultaneous mutations within HPGG (P34G) and HDASH (S158G) motifs does not change its fatty acid distribution pattern (Supplemental Fig. S2), and therefore these changes in *EgΔ5D* do not affect its substrate specificity.

An effective $\Delta 5$ desaturase is required for efficient conversion of DGLA to ARA or ETA to EPA (Fig. 1) in engineered *Y. lipolytica* or other organisms to produce commercially valuable LC-PUFA. We employed two approaches to improve the enzyme activity of the double mutant *EgΔ5D-34G/158G*. The optimization (Fig. 6) of the 43 codons of the 68 amino acids within the N-terminal portion of *EgΔ5D-34G/158G*, *EgΔ5M*, improved substrate conversion (Table 6). This improvement may relate to the rate of translation. Recent reports suggest that sequence at

the beginning of a gene can influence translation, and the mRNA structure at the 5' end of an mRNA can affect protein levels [58, 59]. Next, we substituted the arginine residue at position 347 with the serine which was identified in our original PCR products. Surprisingly, this R347S substitution in codon optimized *EgΔ5MI* further improved substrate conversion (Table 6). These data suggest that some un-conserved amino acids among different $\Delta 5$ desaturases may be good targets for protein evolution to generate improved enzymes. At this stage, the improved *EgΔ5D*, both *EgΔ5M* and *EgΔ5MI* should enable us to engineer *Yarrowia* and other organisms to produce high levels of ARA, EPA and DHA.

In conclusion, our studies suggest that the exact sequences of the HPGG and HDASH motifs are not necessary for the function of *EgΔ5D*. Several amino acids within these two motifs can be changed individually, or simultaneously, without significantly reducing the enzyme activity or altering its substrate specificity. In some cases such as *EgΔ5D-36D*, *EgΔ5D-36S*, and *EgΔ5D-158G* mutants, the $\Delta 5$ desaturase activity can be improved. In order to fully understand the function of *EgΔ5D*, the roles of the $HX_{(2,3)}HH$ and $(H/Q)X_{(2,3)}HH$ motifs as well as the un-conserved amino acids need to be studied in the future.

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References

- Riediger ND, Othman RA, Suh M, Moghadasian MH (2009) A systemic review of the roles of n-3 fatty acids in health and disease. *J Am Diet Assoc* 109:668–679
- Freeman LM (2010) Beneficial effects of omega-3 fatty acids in cardiovascular disease. *J Small Anim Pract* 51:462–470
- Mourek J, Mourek J Jr (2011) Developmentally dependent and different roles of fatty acids OMEGA-6 and OMEGA-3. *Prague Med Rep* 112:81–92
- Mayurasakorn K, Williams JJ, Ten VS, Deckelbaum RJ (2011) Docosahexaenoic acid: brain accretion and roles in neuroprotection after brain hypoxia and ischemia. *Curr Opin Clin Nutr Metab Care* 14:158–167
- Uddin M, Levy BD (2011) Resolvins: natural agonists for resolution of pulmonary inflammation. *Prog Lipid Res* 50:75–88

6. Serhan CN (2007) Resolution phase of inflammation: novel endogenous anti-inflammatory and proresolving lipid mediators and pathways. *Annu Rev Immunol* 25:101–137
7. Kiso Y (2011) Pharmacology in health foods: effects of arachidonic acid and docosahexaenoic acid on the age-related decline in brain and cardiovascular system function. *J Pharmacol Sci* 115:471–475
8. Weitz D, Weintraub H, Fisher E, Schwartzbard AZ (2010) Fish oil for the treatment of cardiovascular disease. *Cardiol Rev* 18:258–263
9. Yokoyama M, Origasa H, Matsuzaki M, Matsuzawa Y, Saito Y, Ishikawa Y, Oikawa S, Sasaki J, Hishida H, Itakura H, Kita T, Kitabatake A, Nakaya N, Sakata T, Shimada K, Shirato K (2007) Effects of eicosapentaenoic acid on major coronary events in hypercholesterolaemic patients (JELIS): a randomised open-label, blinded endpoint analysis. *Lancet* 369:1090–1098
10. Féart C, Peuchant E, Letenneur L, Samieri C, Montagnier D, Fourrier-Reglat A, Barberger-Gateau P (2008) Plasma eicosapentaenoic acid is inversely associated with severity of depressive symptomatology in the elderly: data from the Bordeaux sample of the three-city study. *Am J Clin Nutr* 87:1156–1162
11. Costa LG (2007) Contaminants in fish: risk-benefit considerations. *Arh Hig Rada Toksikol* 58:567–574
12. Streekstra H (2010) Arachidonic acid: fermentative production by *Mortierella* fungi. In: Cohen Z, Ratledge C (eds) *Single cell oils*, 2nd edn. AOCS Press, Urbana
13. Barclay W, Weaver C, Metz J, Hansen J (2010) Development of a docosahexaenoic acid production technology using *Schizochytrium*: historical perspective and update. In: Cohen Z, Ratledge C (eds) *Single cell oils*, 2nd edn. AOCS Press, Urbana
14. Zhu Q, Xue Z, Yadav N, Damude H, Pollak D, Rupert R, Seip J, Hollerbach D, Macool D, Zhang H (2010) Metabolic engineering of an oleaginous yeast for the production of omega-3 fatty acids. In: Cohen Z, Ratledge C (eds) *Single cell oils*, 2nd edn. AOCS Press, Urbana
15. Metz JG, Roessler P, Facciotti D, Levering C, Dittrich F, Lassner M, Valentine R, Lardizabal K, Domergue F, Yamada A, Yazawa K, Knauf V, Browse J (2001) Production of polyunsaturated fatty acids by polyketide synthases in both prokaryotes and eukaryotes. *Science* 293:290–293
16. Wallis JG, Browse J (1999) The $\Delta 8$ -desaturase of *Euglena gracilis*: an alternate pathway for synthesis of 20-carbon polyunsaturated fatty acids. *Arch Biochem Biophys* 365:307–316
17. Pereira SL, Leonard AE, Mukerji P (2003) Recent advances in the study of fatty acid desaturases from animals and lower eukaryotes. *Prostaglandins Leukot Essent Fatty Acids* 68:97–106
18. Qiu X, Hong H, MacKenzie SL (2001) Identification of a delta 4 fatty acid desaturase from *Thraustochytrium* sp. involved in the biosynthesis of docosahexanoic acid by heterologous expression in *Saccharomyces cerevisiae* and *Brassica juncea*. *J Biol Chem* 276:31561–31566
19. Qi B, Fraser T, Mugford S, Dobson G, Sayanova O, Butler JJ, Napier A, Stobart AK, Lazarus CM (2004) Production of very long chain polysaturated omega-3 and omega-6 fatty acids in plants. *Nat Biotechnol* 22:739–745
20. Kajikawa M, Yamato KT, Kohzu Y, Nojiri M, Sakuradani E, Shimizu S, Sakai Y, Fukuzawa H, Ohyama K (2004) Isolation and characterization of delta(6)-desaturase, an ELO-like enzyme and delta(5)-desaturase from the liverwort *Marchantia polymorpha* and production of arachidonic and eicosapentaenoic acids in the methylotrophic yeast *Pichia pastoris*. *Plant Mol Biol* 54:335–352
21. Damude HG, Kinney AJ (2008) Engineering oilseeds to produce nutritional fatty acids. *Physiol Plant* 132:1–10
22. Venegas-Calero M, Sayanova O, Napier JA (2010) An alternative to fish oils: metabolic engineering of oil-seed crops to produce omega-3 long chain polyunsaturated fatty acids. *Prog Lipid Res* 49:108–119
23. Tavares S, Grotkjær T, Obsen T, Haslam RP, Napier JA, Gunnarsson N (2011) Metabolic engineering of *Saccharomyces cerevisiae* for production of eicosapentaenoic acid, using a novel {delta}5-desaturase from *Paramecium tetraurelia*. *Appl Environ Microbiol* 77:1854–1861
24. Michaelson LV, Lazarus CM, Griffiths G, Napier JA, Stobart AK (1998) Isolation of a delta5-fatty acid desaturase gene from *Mortierella alpina*. *J Biol Chem* 273:19055–19059
25. Knutzon DS, Thurmond JM, Huang YS, Chaudhary S, Bobik EG Jr, Chan GM, Kirchner SJ, Mukerji P (1998) Identification of delta5-desaturase from *Mortierella alpina* by heterologous expression in bakers' yeast and canola. *J Biol Chem* 273:29360–29366
26. Meesapyodsuk D, Qiu X (2012) The front-end desaturase: structure, function, evolution and biotechnological use. *Lipids* 47:227–237
27. Sperling P, Heinz E (2001) Desaturases fused to their electron donor. *Eur J Lipid Sci Technol* 103:158–180
28. Napier JA, Michaelson LV, Sayanova O (2003) The role of cytochrome *b*₅ fusion desaturases in the synthesis of polyunsaturated fatty acids. *Prostaglandins Leukot Essent Fatty Acids* 68:135–143
29. Schenkman JB, Jansson I (2003) The many roles of cytochrome *b*₅. *Pharmacol Ther* 97:139–152
30. Stuke JE, McDonough VM, Martin CE (1990) The OLE1 gene of *Saccharomyces cerevisiae* encodes the delta 9 fatty acid desaturase and can be functionally replaced by the rat stearoyl-CoA desaturase gene. *J Biol Chem* 265:20144–20149
31. Shanklin J, Guy JE, Mishra G, Lindqvist Y (2009) Desaturases: emerging models for understanding functional diversification of diiron-containing enzymes. *J Biol Chem* 284:18559–18563
32. Lin YW, Ying TL, Liao LF (2009) Dynamic consequences of mutating the typical HPGG motif of apocytochrome *b*₅ revealed by computer simulation. *Chin Chem Let* 200:631–634
33. Shanklin J, Whittle E, Fox BG (1994) Eight histidine residues are catalytically essential in a membrane-associated iron enzyme, stearoyl-CoA desaturase, and are conserved in alkane hydroxylase and xylene monooxygenase. *Biochemistry* 33:12787–12794
34. Knutzon D, Mukerji P, Huang YS, Thurmond J, Chaudhary S (1999) Methods and compositions for synthesis of long chain poly-unsaturated fatty acids. US Patent 5,972,664
35. Yadav NS, Zhu Q, Zhang H (2009) $\Delta 12$ desaturases suitable for altering levels of polyunsaturated fatty acids in oleaginous yeast. US Patent 7,504,259
36. Macool DJ, Xue Z, Zhu Q (2008) A *Mortierella alpina* *c*_{16/18} fatty acid elongase. US Patent 7,470,532
37. Damude HG, McGonigle B, Zhu Q, Xue Z (2011) Delta-9 elongases and their use in making polyunsaturated fatty acids. US Patent 8,049,062
38. Damude HG, He H, Liao D-I, Zhu Q (2011) Mutant delta-8 desaturase genes engineered by targeted mutagenesis and their use in making polyunsaturated fatty acids. US Patent 8,026,089
39. Sambrook J, Fritsch EF, Maniatis T (1989) *Molecular cloning: a laboratory manual*, 2nd edn. Cold Spring Harbor Laboratory, Cold Spring Harbor
40. Ausubel FM, Brent R, Kingston RE, Moore DD, Seidman JG, Smith JA, Struhl K (2010) *Current protocols in molecular biology*. Wiley, New York
41. Fournier P, Abbas A, Chasles M, Kudla B, Ogrydziak D, Yaver D, Xuan JW, Peito A, Ribe AM, He F, Gaillardin CC (1993) Colocalization of centromeric and replicative functions on autonomously replicating sequences isolated from the yeast *Yarrowia lipolytica*. *Proc Natl Acad Sci USA* 90:4912–4916

42. Hong S-Y, Seip J, Walters-Pollak D, Rupert R, Jackson R, Xue Z, Zhu Q (2011) Engineering *Yarrowia lipolytica* to express secretory invertase with strong FBA1IN promoter. *Yeast* 29:59–72
43. Chen DC, Beckerich JM, Gaillardin C (1997) One-step transformation of the dimorphic yeast *Yarrowia lipolytica*. *Appl Microbiol Biotechnol* 48:232–235
44. Cahoon EB, Ripp KG, Hall SE, Kinney AL (2001) Formation of conjugated Δ^8 , Δ^{10} -double bonds by Δ^{12} -oleic acid desaturase-related enzymes: biosynthetic origin of calendic acid. *J Biol Chem* 276:2637–2643
45. Meyer A, Cirpus P, Ott C, Schlecker R, Zahringer U, Heinz E (2003) Biosynthesis of docosahexaenoic acid in *Euglena gracilis*: biochemical and molecular evidence for the involvement of a delta4-fatty acyl group desaturase. *J Biochem* 42:9779–9788
46. Hong H, Datla N, MacKenzie SL, Qiu X (2002) Isolation and characterization of a $\Delta 5$ FA desaturase from *Pythium irregulare* by heterologous expression in *Saccharomyces cerevisiae* and oilseed crops. *Lipids* 37:863–868
47. Domergue F, Lerchl J, Zahringer U, Heinz E (2002) Cloning and functional characterization of *Phaeodactylum tricorutum* front-end desaturases involved in eicosapentaenoic acid biosynthesis. *Eur J Biochem* 269:4105–4113
48. Damude H, Zhu Q (2007) Delta-8 desaturase and its use in making polyunsaturated fatty acids. US Patent 7,943,823
49. Kobayashi T, Sakaguchi K, Matsuda T, Abe E, Hama Y, Hayashi M, Honda D, Okita Y, Sugimoto S, Okino N, Ito M (2011) Increase of eicosapentaenoic acid in thraustochytrids through thraustochytrid ubiquitin promoter-driven expression of a fatty acid {delta}5 desaturase gene. *Appl Environ Microbiol* 77:3870–3876
50. Lederer F (1994) The cytochrome b4-fold: an adaptable module. *Biochimie* 76:674–692
51. Sayanova O, Shewry PR, Napier JA (1999) Histidine-41 of the cytochrome b₅ domain of the borage delta6 fatty acid desaturase is essential for enzyme activity. *Plant Physiol* 121:641–646
52. Guillou H, D'Andrea S, Rioux V, Barnouin R, Dalaine S, Pedrono F, Jan S, Legrand P (2004) Distinct roles of endoplasmic reticulum cytochrome b₅ and fused cytochrome b₅-like domain for rat $\Delta 6$ -desaturase activity. *J Lipid Res* 45:32–40
53. Hongsthong A, Subudhi S, Sirijuntarut M, Kurdrud P, Cheevadhanarak S, Tanticharoen M (2006) Revealing the complementation of ferredoxin by cytochrome b₅ in the *Spirulina- $\Delta 6$* -desaturation reaction by N-terminal fusion and co-expression of the fungal-cytochrome b₅ domain and *Spirulina- $\Delta 6$* -acyl-lipid desaturase. *Appl Microbiol Biotechnol* 72:1192–1201
54. Gostincar C, Turk M, Gunde-Cimerman N (2010) The evolution of fatty acid desaturases and cytochrome b₅ in eukaryotes. *J Membr Biol* 233:63–72
55. Ratledge C (2010) Single cell oils for the 21st century. In: Cohen Z, Ratledge C (eds) *Single cell oils*, 2nd edn. AOCS Press, Urbana
56. Beopoulos A, Nicaud JM, Gaillardin C (2011) An overview of lipid metabolism in yeasts and its impact on biotechnological processes. *Appl Microbiol Biotechnol* 90:1193–1206
57. Sabirova JS, Haddouche R, Van Bogaert IN, Mulaa F, Verstraete W, Timmis KN, Schmidt-Dannert C, Nicaud JM, Soetaert W (2011) The 'LipoYeasts' project: using the oleaginous yeast *Yarrowia lipolytica* in combination with specific bacterial genes for the bioconversion of lipids, fats and oils into high-value products. *Microb Biotechnol* 4:47–54
58. Kudla G, Murray AW, Tollervey D, Plotkin JB (2009) Coding sequence determinants of gene expression in *Escherichia coli*. *Science* 324:255–258
59. Kahali B, Ahmad S, Ghosh TC (2011) Selective constraints in yeast genes with differential expressivity: codon pair usage and mRNA stability perspectives. *Gene* 481:76–82