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(54) **Title:** DELTA 9 ELONGASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

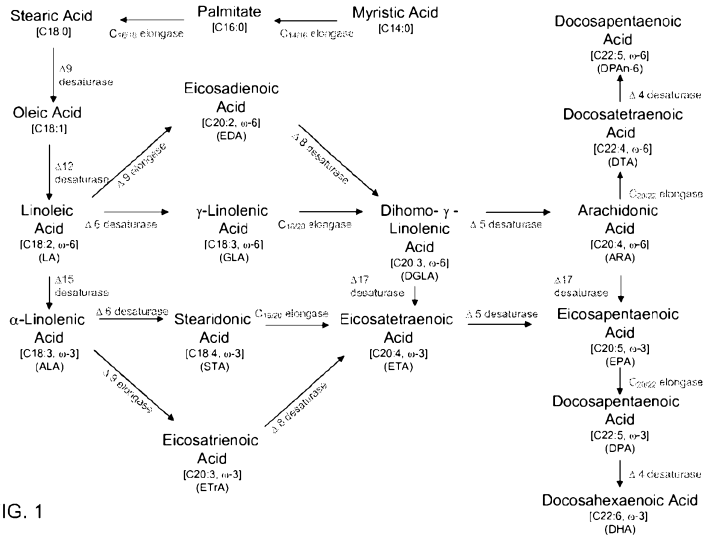

FIG. 1

(57) **Abstract:** The present invention relates to Δ9 elongases, which have the ability to convert linoleic acid (LA; 18:2 ϖ-6) to eicosadienoic acid (EDA; 20:2 ϖ-6) and/or a-linolenic acid (ALA; 18:3 ϖ-3) to eicosatrienoic acid (ETrA; 20:3 ϖ-3). Isolated nucleic acid fragments and recombinant constructs comprising such fragments encoding Δ9 elongases along with a method of making long-chain polyunsaturated fatty acids (PUFAs) using these Δ9 elongases in oleaginous yeast are disclosed.

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TITLE

Δ9 ELONGASES AND THEIR USE IN MAKING POLYUNSATURATED FATTY ACIDS

This application claims the benefit of U.S. Provisional Application No. 60/911,925, filed April 16, 2007, which is herein incorporated by reference in its entirety.

FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this invention pertains to the identification of polynucleotide sequences encoding Δ9 fatty acid elongases and the use of these elongases in making long-chain polyunsaturated fatty acids (PUFAs).

BACKGROUND OF THE INVENTION

Today, a variety of different hosts including plants, algae, fungi, stramenopiles and yeast are being investigated as means for commercial PUFA production. Genetic engineering has demonstrated that the natural abilities of some hosts (even those natively limited to linoleic acid (LA; 18:2 ω-6) and α-linolenic acid (ALA; 18:3 ω-3) fatty acid production) can be substantially altered to result in high-level production of various long-chain ω-3/ω-6 PUFAs. Whether this is the result of natural abilities or recombinant technology, production of arachidonic acid (ARA; 20:4 ω-6), eicosapentaenoic acid (EPA; 20:5 ω-3) and docosahexaenoic acid (DHA; 22:6 ω-3) may require expression of a Δ9 elongase.

Most Δ9 elongase enzymes identified so far have the ability to convert both LA to eicosadienoic acid (EDA; 20:2 ω-6) and ALA to eicosatrienoic acid (ETrA; 20:3 ω-3) (wherein dihomo-γ–linolenic acid (DGLA; 20:3 ω-6) and eicosatetraenoic acid (ETA; 20:4 ω-3) are subsequently synthesized from EDA and ETrA, respectively, following reaction with a Δ8 desaturase; ARA and EPA are subsequently synthesized from DGLA and ETA, respectively, following reaction with a Δ5 desaturase; and, DHA synthesis requires subsequent expression of an additional $C_{20/22}$ elongase and a Δ4 desaturase).

In spite of the need for new methods for the production of ARA, EPA and DHA, few Δ9 elongase enzymes have been identified. A Δ9 elongase from *Isochrysis galbana* is publicly available (described in GenBank Accession No.

AAL37626, as well as PCT Publications No. WO 02/077213, No. WO 2005/083093, No. WO 2005/012316 and No. WO 2004/057001). PCT Publications No. WO 2007/061845 and No. WO 2007/061742 (Applicants' Assignee's co-pending applications), disclose Δ9 elongases from *Euglena gracilis* and *Eutreptiella* sp. CCMP389, as well as Δ9 elongase motifs.

Thus, there is need for the identification and isolation of additional genes encoding Δ9 elongases that will be suitable for heterologous expression in a variety of host organisms for use in the production of ω-3/ω-6 fatty acids.

Applicants have solved the stated problem by isolating genes encoding Δ9 fatty acid elongases from *Euglena anabaena*.

SUMMARY OF THE INVENTION

The present invention relates to new genetic constructs encoding polypeptides having Δ9 elongase activity, and their use in algae, bacteria, yeast, euglenoids, stramenopiles and fungi for the production of PUFAs. Accordingly the invention provides a transformed microbial host cell comprising a non-native polynucleotide encoding a polypeptide having delta-9 elongase activity, wherein the polypeptide comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:13 or SEQ ID NO:14 , based on the Clustal V method of alignment..

In another embodiment the invention provides a method for the production of eicosadienoic acid comprising:

a) providing a microbial host cell comprising:
   (i) a recombinant nucleotide molecule encoding a Δ9 elongase polypeptide having at least 90% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14; and,
   (ii) a source of linoleic acid;
b) growing the microbial host cell of step (a) under conditions wherein the polynucleotide encoding the polypeptide having Δ9 elongase is expressed and the linoleic acid is converted to eicosadienoic acid; and,
c) optionally recovering the eicosadienoic acid of step (b).

In an additional embodiment the invention provides a method for the production of eicosatrienoic acid comprising:

a) providing a microbial host cell comprising:

(i) a recombinant nucleotide molecule encoding a Δ9 elongase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14; and,

(ii) a source of α-linolenic acid;

b) growing the microbial host cell of step (a) under conditions wherein the nucleic acid fragment encoding the Δ9 elongase polypeptide is expressed and the α-linolenic acid is converted to eicosatrienoic acid; and,

c) optionally recovering the eicosatrienoic acid of step (b).

In another embodiment the invention provides an isolated nucleic acid molecule which encodes a Δ9 elongase as set forth in SEQ ID NO:26 wherein at least 98 codons are codon-optimized for expression in *Yarrowia* sp.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE LISTINGS

FIG. 1 is a representative ω-3 and ω-6 fatty acid biosynthetic pathway providing for the conversion of myristic acid through various intermediates to DHA.

FIG. 2 shows a chromatogram of the lipid profile of an *Euglena anabaena* cell extract as described in the Examples.

FIG. 3 provides plasmid maps for the following: (A) pY115 (SEQ ID NO:19); (B) pY159 (SEQ ID NO:23); (C) pY173 (SEQ ID NO:24); and, (D) pY174 (SEQ ID NO:25).

FIGs. 4A and 4B show a comparison of the nucleotide sequences of EaD9Elo (SEQ ID NO:11) and EaD9ES (SEQ ID NO:26).

FIG. 5 provides plasmid maps for the following: (A) pEaD9ES (SEQ ID NO:28); and, (B) pZUFmEaD9eS (SEQ ID NO:29).

The invention can be more fully understood from the following detailed description and the accompanying sequence descriptions, which form a part of this application.

The following sequences comply with 37 C.F.R. §1.821-1.825 ("Requirements for Patent Applications Containing Nucleotide Sequences and/or Amino Acid Sequence Disclosures - the Sequence Rules") and are consistent with World Intellectual Property Organization (WIPO) Standard ST.25 (1998) and the sequence listing requirements of the EPO and PCT (Rules 5.2 and 49.5(a-bis), and Section 208 and Annex C of the Administrative Instructions). The symbols and format used for nucleotide and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NOs:1-4, 9-19 and 22-29 are ORFs encoding genes or proteins (or portions thereof), or plasmids, as identified in Table 1.

Table 1

Summary Of Nucleic Acid And Protein SEQ ID Numbers

Description and Abbreviation	**Nucleic acid SEQ ID NO.**	**Protein SEQ ID NO.**
Euglena anabaena Δ9 elongase cDNA sequence ("EaD9Elo1")	1 (1129 bp)	--
Euglena anabaena Δ9 elongase cDNA sequence ("EaD9Elo2")	2 (1145 bp)	--
Euglena gracilis Δ9 elongase coding sequence ("EgD9e")	3 (774 bp)	4 (258 AA)
Plasmid pLF121-1	9 (3668 bp)	--
Plasmid pLF121-2	10 (3684 bp)	--

Euglena anabaena Δ9 elongase coding sequence ("EaD8Des1 CDS" or "EaD9Elo1", respectively)	11 (774 bp)	13 (258 AA)
Euglena anabaena Δ9 elongase coding sequence ("EaD8Des2 CDS" or "EaD9Elo2", respectively)	12 (774 bp)	14 (258 AA)
Plasmid pKR906	15 (4311 bp)	--
Isochrysis galbana Δ9 elongase (IgD9e)	--	16 (263 AA)
Plasmid pDMW263	17 (9472 bp)	--
Plasmid pDMW237	18 (7879 bp)	--
Plasmid pY115	19 (7783 bp)	--
Plasmid pY158	22 (6992 bp)	--
Plasmid pY159	23 (8707 bp)	--
Plasmid pY173	24 (8219 bp)	--
Plasmid pY174	25 (8235 bp)	
Synthetic Δ9 elongase, derived from *Euglena anabaena,* codon-optimized for expression in *Yarrowia lipolytica* ("EaD9eS")	26 (774 bp)	27 (258 AA)
Plasmid pEaD9ES	28 (3497 bp)	
Plasmid pZUFmEaD9eS	29 (7769 bp)	--

SEQ ID NOs:5 and 6 correspond to oligonucleotides oEugEL1-1 and oEugEL1-2, respectively, used for amplification of the *Euglena gracilis* Δ9 elongase.

SEQ ID NOs:7 and 8 correspond to the M13F universal primer and primer M13-28Rev, respectively, used for end-sequencing of *Euglena anabaena* DNA inserts.

SEQ ID NOs:20 and 21 correspond to primers oYFBA1 and oYFBA1-6, respectively, used to amplify the FBAINm promoter from plasmid pY115.

DETAILED DESCRIPTION OF THE INVENTION

New *Euglena anabaena* Δ9 elongase enzymes and genes encoding the same that may be used for the manipulation of biochemical pathways for the production of healthful PUFAs are disclosed herein.

PUFAs, or derivatives thereof, are used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing intravenous

feeding or for preventing or treating malnutrition. Alternatively, the purified PUFAs (or derivatives thereof) may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount for dietary supplementation. The PUFAs may also be incorporated into infant formulas, nutritional supplements or other food products and may find use as anti-inflammatory or cholesterol lowering agents. Optionally, the compositions may be used for pharmaceutical use (human or veterinary).

Definitions

In the context of this disclosure, a number of terms and abbreviations are used. The following definitions are provided.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

"American Type Culture Collection" is abbreviated ATCC.

"Polyunsaturated fatty acid(s)" is abbreviated PUFA(s).

"Triacylglycerols" are abbreviated TAGs.

The term "invention" or "present invention" as used herein is not meant to be limiting to any one specific embodiment of the invention but applies generally to any and all embodiments of the invention as described in the claims and specification.

As used herein and in the appended claims, the singular forms "a", "an", and "the" include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to "a plant" includes a plurality of such plants, reference to "a cell" includes one or more cells and equivalents thereof known to those skilled in the art, and so forth.

The term "fatty acids" refers to long-chain aliphatic acids (alkanoic acids) of varying chain lengths, from about C_{12} to C_{22} (although both longer and shorter chain-length acids are known). The predominant chain lengths are between C_{16} and C_{22}. Additional details concerning the differentiation between "saturated fatty acids" versus "unsaturated fatty acids", "monounsaturated fatty acids" versus "polyunsaturated fatty acids" (or "PUFAs"), and "omega-6 fatty acids" (ω-6 or *n*-6) versus "omega-3 fatty acids" (ω-3 or *n*-3) are provided in U.S. Patent 7,238,482.

Fatty acids are described herein by a simple notation system of "X:Y", where X is the total number of carbon (C) atoms in the particular fatty acid and Y is the number of double bonds. The number following the fatty acid designation indicates

the position of the double bond from the carboxyl end of the fatty acid with the "c" affix for the *cis*-configuration of the double bond (e.g., palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1, 9c), petroselinic acid (18:1, 6c), LA (18:2, 9c,12c), GLA (18:3, 6c,9c,12c) and ALA (18:3, 9c,12c,15c)). Unless otherwise specified, 18:1, 18:2 and 18:3 refer to oleic, LA and ALA fatty acids, respectively. If not specifically written as otherwise, double bonds are assumed to be of the *cis* configuration. For instance, the double bonds in 18:2 (9,12) would be assumed to be in the *cis* configuration.

Nomenclature used to describe PUFAs in the present disclosure is shown below in Table 2. In the column titled "Shorthand Notation", the omega-reference system is used to indicate the number of carbons, the number of double bonds and the position of the double bond closest to the omega carbon, counting from the omega carbon (which is numbered 1 for this purpose). The remainder of the Table summarizes the common names of ω-3 and ω-6 fatty acids and their precursors, the abbreviations that will be used throughout the remainder of the specification, and each compounds' chemical name.

Table 2

Nomenclature of Polyunsaturated Fatty Acids and Precursors

Common Name	Abbreviation	Chemical Name	Shorthand Notation
Myristic	--	tetradecanoic	14:0
Palmitic	PA or Palmitate	hexadecanoic	16:0
Palmitoleic	--	9-hexadecenoic	16:1
Stearic	--	octadecanoic	18:0
Oleic	--	*cis*-9-octadecenoic	18:1
Linoleic	LA	*cis*-9,12-octadecadienoic	18:2 ω-6
γ-Linolenic	GLA	*cis*-6,9,12-octadecatrienoic	18:3 ω-6
Eicosadienoic	EDA	*cis*-11,14-eicosadienoic	20:2 ω-6
Dihomo-γ-linolenic	DGLA	*cis*-8,11,14-eicosatrienoic	20:3 ω-6
Sciadonic	SCI	*cis*-5,11,14-eicosatrienoic	20:3b ω-6
Arachidonic	ARA	*cis*-5,8,11,14-eicosatetraenoic	20:4 ω-6
α-Linolenic	ALA	*cis*-9,12,15-octadecatrienoic	18:3 ω-3

Stearidonic	STA	*cis*-6,9,12,15-octadecatetraenoic	18:4 ω-3
Eicosatrienoic	ETrA or ERA	*cis*-11,14,17-eicosatrienoic	20:3 ω-3
Eicosa-tetraenoic	ETA	*cis*-8,11,14,17-eicosatetraenoic	20:4 ω-3
Juniperonic	JUP	*cis*-5,11,14,17-eicosatrienoic	20:4b ω-3
Eicosa-pentaenoic	EPA	*cis*-5,8,11,14,17-eicosapentaenoic	20:5 ω-3
Docosatrienoic	DRA	*cis*-10,13,16-docosatrienoic	22:3 ω-6
Docosa-tetraenoic	DTA	*cis*-7,10,13,16-docosatetraenoic	22:4 ω-6
Docosa-pentaenoic	DPAn-6	*cis*-4,7,10,13,16-docosapentaenoic	22:5 ω-6
Docosa-pentaenoic	DPA	*cis*-7,10,13,16,19-docosapentaenoic	22:5 ω-3
Docosa-hexaenoic	DHA	*cis*-4,7,10,13,16,19-docosahexaenoic	22:6 ω-3

The terms "triacylglycerol", "oil" and "TAGs" refer to neutral lipids composed of three fatty acyl residues esterified to a glycerol molecule (and such terms will be used interchangeably throughout the present disclosure herein). Such oils can contain long chain PUFAs, as well as shorter saturated and unsaturated fatty acids and longer chain saturated fatty acids. Thus, "oil biosynthesis" generically refers to the synthesis of TAGs in the cell.

"Percent (%) PUFAs in the total lipid and oil fractions" refers to the percent of PUFAs relative to the total fatty acids in those fractions. The term "total lipid fraction" or "lipid fraction" both refer to the sum of all lipids (i.e., neutral and polar) within an oleaginous organism, thus including those lipids that are located in the phosphatidylcholine (PC) fraction, phosphatidyletanolamine (PE) fraction and triacylglycerol (TAG or oil) fraction. However, the terms "lipid" and "oil" will be used interchangeably throughout the specification.

A metabolic pathway, or biosynthetic pathway, in a biochemical sense, can be regarded as a series of chemical reactions occurring within a cell, catalyzed by enzymes, to achieve either the formation of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway (then called a flux generating step). Many of these pathways are elaborate, and involve a step by step

modification of the initial substance to shape it into a product having the exact chemical structure desired.

The term "PUFA biosynthetic pathway" refers to a metabolic process that converts oleic acid to ω-6 fatty acids such as LA, EDA, GLA, DGLA, ARA, DRA, DTA and DPAn-6 and ω-3 fatty acids such as ALA, STA, ETrA, ETA, EPA, DPA and DHA. This process is well described in the literature (e.g., see PCT Publication No. WO 2006/052870). Simplistically, this process involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds, via a series of special desaturation and elongation enzymes (i.e., "PUFA biosynthetic pathway enzymes") present in the endoplasmic reticulim membrane. More specifically, "PUFA biosynthetic pathway enzyme" refers to any of the following enzymes (and genes which encode said enzymes) associated with the biosynthesis of a PUFA, including: a Δ9 elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase, a $C_{20/22}$ elongase, a Δ4 desaturase, a Δ5 desaturase, a Δ6 desaturase, a Δ12 desaturase, a Δ15 desaturase, a Δ17 desaturase, a Δ9 desaturase and/or a Δ8 desaturase.

The term "ω-3/ω-6 fatty acid biosynthetic pathway" refers to a set of genes which, when expressed under the appropriate conditions encode enzymes that catalyze the production of either or both ω-3 and ω-6 fatty acids. Typically the genes involved in the ω-3/ω-6 fatty acid biosynthetic pathway encode PUFA biosynthetic pathway enzymes. A representative pathway is illustrated in FIG. 1, providing for the conversion of myristic acid through various intermediates to DHA, which demonstrates how both ω-3 and ω-6 fatty acids may be produced from a common source. The pathway is naturally divided into two portions where one portion will generate ω-3 fatty acids and the other portion, ω-6 fatty acids.

The term "functional" as used herein in context with the ω-3/ω-6 fatty acid biosynthetic pathway means that some (or all) of the genes in the pathway express active enzymes, resulting in *in vivo* catalysis or substrate conversion. It should be understood that "ω-3/ω-6 fatty acid biosynthetic pathway" or "functional ω-3/ω-6 fatty acid biosynthetic pathway" does not imply that all the PUFA biosynthetic pathway enzyme genes are required, as a number of fatty acid products will only require the expression of a subset of the genes of this pathway.

The term "Δ6 desaturase/ Δ6 elongase pathway" will refer to a PUFA biosynthetic pathway that minimally includes at least one Δ6 desaturase and at least one $C_{18/20}$ elongase (also referred to as a Δ6 elongase), thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively, with GLA and/or STA as intermediate fatty acids. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized.

The term "Δ9 elongase/ Δ8 desaturase pathway" will refer to a PUFA biosynthetic pathway that minimally includes at least one Δ9 elongase and at least one Δ8 desaturase, thereby enabling biosynthesis of DGLA and/or ETA from LA and ALA, respectively, with EDA and/or ETrA as intermediate fatty acids. With expression of other desaturases and elongases, ARA, EPA, DPA and DHA may also be synthesized.

The term "intermediate fatty acid" refers to any fatty acid produced in a fatty acid metabolic pathway that can be further converted to an intended product fatty acid in this pathway by the action of other metabolic pathway enzymes. For instance, when EPA is produced using the Δ9 elongase/Δ8 desaturase pathway, EDA, ETrA, DGLA, ETA and ARA can be produced and are considered "intermediate fatty acids" since these fatty acids can be further converted to EPA via action of other metabolic pathway enzymes.

The term "by-product fatty acid" refers to any fatty acid produced in a fatty acid metabolic pathway that is not the intended fatty acid product of the pathway nor an "intermediate fatty acid" of the pathway. For instance, when EPA is produced using the Δ9 elongase/Δ8 desaturase pathway, sciadonic acid (SCI) and juniperonic acid (JUP) also can be produced by the action of a Δ5 desaturase on either EDA or ETrA, respectively. They are considered to be "by-product fatty acids" since neither can be further converted to EPA by the action of other metabolic pathway enzymes.

The term "desaturase" refers to a polypeptide that can desaturate, i.e., introduce a double bond, in one or more fatty acids to produce a fatty acid or precursor of interest. Despite use of the omega-reference system throughout the specification to refer to specific fatty acids, it is more convenient to indicate the activity of a desaturase by counting from the carboxyl end of the substrate using the delta-system. Desaturases of interest include, for example: (1) Δ8 desaturases that desaturate a fatty acid between the eighth and ninth carbon atom numbered from

the carboxyl-terminal end of the molecule and that can, for example, catalyze the conversion of EDA to DGLA and/or ETrA to ETA; (2) Δ5 desaturases that catalyze the conversion of DGLA to ARA and/or ETA to EPA; (3) Δ6 desaturases that catalyze the conversion of LA to GLA and/or ALA to STA; (4) Δ4 desaturases that catalyze the conversion of DPA to DHA and/or DTA to DPAn-6; (5) Δ12 desaturases that catalyze the conversion of oleic acid to LA; (6) Δ15 desaturases that catalyze the conversion of LA to ALA and/or GLA to STA; (7) Δ17 desaturases that catalyze the conversion of ARA to EPA and/or DGLA to ETA; and, (8) Δ9 desaturases that catalyze the conversion of palmitic acid to palmitoleic acid (16:1) and/or stearic acid to oleic acid (18:1). In the art, Δ15 and Δ17 desaturases are also occasionally referred to as "omega-3 desaturases", "w-3 desaturases" and/or "ω-3 desaturases", based on their ability to convert ω-6 fatty acids into their ω-3 counterparts (e.g., conversion of LA into ALA and ARA into EPA, respectively). In some embodiments, it may be desirable to empirically determine the specificity of a particular fatty acid desaturase by transforming a suitable host with the gene for the fatty acid desaturase and determining its effect on the fatty acid profile of the host.

For the purposes herein, an enzyme catalyzing the first condensation reaction (i.e., conversion of malonyl-CoA and long-chain acyl-CoA to □-ketoacyl-CoA) will be referred to generically as an "elongase". In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree of unsaturation. Accordingly, elongases can have different specificities. For example, a $C_{14/16}$ elongase will utilize a C_{14} substrate (e.g., myristic acid), a $C_{16/18}$ elongase will utilize a C_{16} substrate (e.g., palmitate), a $C_{18/20}$ elongase (also known as a Δ6 elongase as the terms can be used interchangeably) will utilize a C_{18} substrate (e.g., GLA, STA) and a $C_{20/22}$ elongase will utilize a C_{20} substrate (e.g., ARA, EPA). In like manner, and of particular interest herein, a "Δ9 elongase" catalyzes the conversion of LA to EDA and/or ALA to ETrA. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions. Thus, for example, a Δ9 elongase may also act as a $C_{16/18}$ elongase, $C_{18/20}$ elongase and/or $C_{20/22}$ elongase and may have alternate, but not preferred, specificities for Δ5 and Δ6 fatty acids such as EPA and/or GLA, respectively. In preferred embodiments, it may be desirable to

empirically determine the specificity of a fatty acid elongase by transforming a suitable host with the gene for the fatty acid elongase and determining its effect on the fatty acid profile of the host. Elongase systems generally comprise four enzymes that are responsible for elongation of a fatty acid carbon chain to produce a fatty acid that is two carbons longer than the fatty acid substrate that the elongase system acts upon. More specifically, the process of elongation occurs in association with fatty acid synthase, whereby CoA is the acyl carrier (Lassner et al., *Plant Cell,* 8:281-292 (1996)). In the first step, which has been found to be both substrate-specific and also rate-limiting, malonyl-CoA is condensed with a long-chain acyl-CoA to yield carbon dioxide (CO_2) and a β-ketoacyl-CoA (where the acyl moiety has been elongated by two carbon atoms). Subsequent reactions include reduction to β-hydroxyacyl-CoA, dehydration to an enoyl-CoA and a second reduction to yield the elongated acyl-CoA. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA, LA to EDA, ALA to ETrA, ARA to DTA and EPA to DPA.

For the purposes herein, the term "EaD9Elo1" refers to a Δ9 elongase enzyme (SEQ ID NO:13) isolated from *Euglena anabaena,* encoded by SEQ ID NO:11 herein. The term "EaD9Elo2" refers to a Δ9 elongase enzyme (SEQ ID NO:14) isolated from *E. anabaena,* encoded by SEQ ID NO:12 herein. Likewise, the term "EaD9eS" refers to a synthetic Δ9 elongase derived from *E. anabaena* that is codon-optimized for expression in *Yarrowia lipolytica* (i.e., SEQ ID NOs:26 and 27).

The terms "conversion efficiency" and "percent substrate conversion" refer to the efficiency by which a particular enzyme (e.g., an elongase) can convert substrate to product. The conversion efficiency is measured according to the following formula: ([product]/[substrate + product])*100, where 'product' includes the immediate product and all products in the pathway derived from it.

The term "oleaginous" refers to those organisms that tend to store their energy source in the form of lipid (Weete, In: Fungal Lipid Biochemistry, 2nd Ed., Plenum, 1980). Within oleaginous microorganisms the cellular oil or TAG content generally follows a sigmoid curve, wherein the concentration of lipid increases until it reaches a maximum at the late logarithmic or early stationary growth phase and then gradually decreases during the late stationary and death phases

(Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)). It is not uncommon for oleaginous microorganisms to accumulate in excess of about 25% of their dry cell weight as oil.

The term "oleaginous yeast" refers to those microorganisms classified as yeasts that make oil. Examples of oleaginous yeast include, but are no means limited to, the following genera: *Yarrowia, Candida, Rhodotorula, Rhodosporidium*, *Cryptococcus, Trichosporon* and *Lipomyces*.

The term "Euglenophyceae" refers to a group of unicellular colorless or photosynthetic flagellates ("euglenoids") found living in freshwater, marine, soil, and parasitic environments. The class is characterized by solitary unicells, wherein most are free-swimming and have two flagella (one of which may be nonemergent) arising from an anterior invagination known as a reservoir. Photosynthetic euglenoids contain one to many chloroplasts, which vary from minute disks to expanded plates or ribbons. Colorless euglenoids depend on osmotrophy or phagotrophy for nutrient assimilation. About 1000 species have been described and classified into about 40 genera and 6 orders. Examples of Euglenophyceae include, but are no means limited to, the following genera: *Euglena*, *Eutreptiella* and *Tetruetreptia.*

As used herein, "nucleic acid" means a polynucleotide and includes single or double-stranded polymer of deoxyribonucleotide or ribonucleotide bases. Nucleic acids may also include fragments and modified nucleotides. Thus, the terms "polynucleotide", "nucleic acid sequence", "nucleotide sequence" or "nucleic acid fragment" are used interchangeably and refer to a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. A polynucleotide in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA, synthetic DNA, or mixtures thereof. Nucleotides (usually found in their 5'-monophosphate form) are referred to by their single letter designation as follows: "A" for adenylate or deoxyadenylate (for RNA or DNA, respectively), "C" for cytidylate or deosycytidylate, "G" for guanylate or deoxyguanylate, "U" for uridlate, "T" for deosythymidylate, "R" for purines (A or G), "Y" for pyrimidiens (C or T), "K" for G or T, "H" for A or C or T, "I" for inosine, and "N" for any nucleotide.

The term “conserved domain” or “motif” means a set of amino acids conserved at specific positions along an aligned sequence of evolutionarily related proteins. While amino acids at other positions can vary between homologous proteins, amino acids that are highly conserved at specific positions indicate amino acids that are essential in the structure, the stability, or the activity of a protein. Because they are identified by their high degree of conservation in aligned sequences of a family of protein homologues, they can be used as identifiers, or “signatures”, to determine if a protein with a newly determined sequence belongs to a previously identified protein family. PCT Publications No. WO 2007/061845 and No. WO 2007/061742 describe seven distinct motifs that are associated with Δ9 elongases.

The terms “homology”, “homologous”, “substantially similar” and “corresponding substantially” are used interchangeably herein. They refer to nucleic acid fragments wherein changes in one or more nucleotide bases do not affect the ability of the nucleic acid fragment to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the nucleic acid fragments of the instant invention such as deletion or insertion of one or more nucleotides that do not substantially alter the functional properties of the resulting nucleic acid fragment relative to the initial, unmodified fragment. It is therefore understood, as those skilled in the art will appreciate, that the invention encompasses more than the specific exemplary sequences.

Moreover, the skilled artisan recognizes that substantially similar nucleic acid sequences encompassed by this invention are also defined by their ability to hybridize (under moderately stringent conditions, e.g., 0.5X SSC, 0.1% SDS, 60 °C) with the sequences exemplified herein, or to any portion of the nucleotide sequences disclosed herein and which are functionally equivalent to any of the nucleic acid sequences disclosed herein. Stringency conditions can be adjusted to screen for moderately similar fragments, such as homologous sequences from distantly related organisms, to highly similar fragments, such as genes that duplicate functional enzymes from closely related organisms. Post-hybridization washes determine stringency conditions.

The term "selectively hybridizes" includes reference to hybridization, under stringent hybridization conditions, of a nucleic acid sequence to a specified nucleic

acid target sequence to a detectably greater degree (e.g., at least 2-fold over background) than its hybridization to non-target nucleic acid sequences and to the substantial exclusion of non-target nucleic acids. Selectively hybridizing sequences typically have about at least 80% sequence identity, or 90% sequence identity, up to and including 100% sequence identity (i.e., fully complementary) with each other.

The term "stringent conditions" or “stringent hybridization conditions” includes reference to conditions under which a probe will selectively hybridize to its target sequence. Stringent conditions are sequence-dependent and will be different in different circumstances. By controlling the stringency of the hybridization and/or washing conditions, target sequences can be identified which are 100% complementary to the probe (homologous probing). Alternatively, stringency conditions can be adjusted to allow some mismatching in sequences so that lower degrees of similarity are detected (heterologous probing). Generally, a probe is less than about 1000 nucleotides in length, optionally less than 500 nucleotides in length.

Typically, stringent conditions will be those in which the salt concentration is less than about 1.5 M Na ion, typically about 0.01 to 1.0 M Na ion concentration (or other salts) at pH 7.0 to 8.3 and the temperature is at least about 30 °C for short probes (e.g., 10 to 50 nucleotides) and at least about 60 °C for long probes (e.g., greater than 50 nucleotides). Stringent conditions may also be achieved with the addition of destabilizing agents such as formamide. Exemplary low stringency conditions include hybridization with a buffer solution of 30 to 35% formamide, 1 M NaCl, 1% SDS (sodium dodecyl sulphate) at 37 °C, and a wash in 1X to 2X SSC (20X SSC = 3.0 M NaCl/0.3 M trisodium citrate) at 50 to 55 °C. Exemplary moderate stringency conditions include hybridization in 40 to 45% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.5X to 1X SSC at 55 to 60 °C. Exemplary high stringency conditions include hybridization in 50% formamide, 1 M NaCl, 1% SDS at 37 °C, and a wash in 0.1X SSC at 60 to 65 °C.

Specificity is typically the function of post-hybridization washes, the important factors being the ionic strength and temperature of the final wash solution. For DNA-DNA hybrids, the T_m can be approximated from the equation of Meinkoth et al., *Anal. Biochem.,* 138:267-284 (1984): T_m = 81.5 °C + 16.6 (log M) + 0.41 (%GC) - 0.61 (% form) - 500/L; where M is the molarity of monovalent cations, %GC is the percentage of guanosine and cytosine nucleotides in the DNA, % form is the

percentage of formamide in the hybridization solution, and L is the length of the hybrid in base pairs. The T_m is the temperature (under defined ionic strength and pH) at which 50% of a complementary target sequence hybridizes to a perfectly matched probe. T_m is reduced by about 1°C for each 1% of mismatching; thus, T_m, hybridization and/or wash conditions can be adjusted to hybridize to sequences of the desired identity. For example, if sequences with $\geq$90% identity are sought, the T_m can be decreased 10 °C. Generally, stringent conditions are selected to be about 5 °C lower than the thermal melting point (T_m) for the specific sequence and its complement at a defined ionic strength and pH. However, severely stringent conditions can utilize a hybridization and/or wash at 1, 2, 3, or 4 °C lower than the thermal melting point (T_m); moderately stringent conditions can utilize a hybridization and/or wash at 6, 7, 8, 9, or 10 °C lower than the thermal melting point (T_m); and, low stringency conditions can utilize a hybridization and/or wash at 11, 12, 13, 14, 15, or 20 °C lower than the thermal melting point (T_m). Using the equation, hybridization and wash compositions, and desired T_m, those of ordinary skill will understand that variations in the stringency of hybridization and/or wash solutions are inherently described. If the desired degree of mismatching results in a T_m of less than 45 °C (aqueous solution) or 32 °C (formamide solution), it is preferred to increase the SSC concentration so that a higher temperature can be used. An extensive guide to the hybridization of nucleic acids is found in Tijssen, *Laboratory Techniques in Biochemistry and Molecular Biology--Hybridization with Nucleic Acid Probes,* Part I, Chapter 2 "Overview of principles of hybridization and the strategy of nucleic acid probe assays", Elsevier, New York (1993); and *Current Protocols in Molecular Biology*, Chapter 2, Ausubel et al., Eds., Greene Publishing and Wiley-Interscience, New York (1995). Hybridization and/or wash conditions can be applied for at least 10, 30, 60, 90, 120 or 240 minutes.

"Sequence identity" or "identity" in the context of nucleic acid or polypeptide sequences refers to the nucleic acid bases or amino acid residues in two sequences that are the same when aligned for maximum correspondence over a specified comparison window.

Thus, "percentage of sequence identity" refers to the value determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide or polypeptide sequence in the comparison window

may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the results by 100 to yield the percentage of sequence identity. Useful examples of percent sequence identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90% or 95%, or any integer percentage from 50% to 100%. These identities can be determined using any of the programs described herein.

Sequence alignments and percent identity or similarity calculations may be determined using a variety of comparison methods designed to detect homologous sequences including, but not limited to, the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

The "Clustal V method of alignment" corresponds to the alignment method labeled Clustal V (described by Higgins and Sharp, *CABIOS*, 5:151-153 (1989); Higgins, D.G. et al., *Comput. Appl. Biosci.*, 8:189-191 (1992)) and found in the MegAlign™ program of the LASERGENE bioinformatics computing suite (*supra*). For multiple alignments, the default values correspond to GAP PENALTY=10 and GAP LENGTH PENALTY=10. Default parameters for pairwise alignments and calculation of percent identity of protein sequences using the Clustal V method are KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5. For nucleic acids these parameters are KTUPLE=2, GAP PENALTY=5, WINDOW=4 and DIAGONALS SAVED=4. After alignment of the sequences using the Clustal V program, it is possible to obtain a "percent identity" by viewing the "sequence distances" table in the same program.

"BLASTN method of alignment" is an algorithm provided by the National Center for Biotechnology Information (NCBI) to compare nucleotide sequences using default parameters.

It is well understood by one skilled in the art that many levels of sequence identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar function or activity. Useful examples of percent identities include, but are not limited to, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, or 95%, or any integer percentage from 50% to 100%. Indeed, any integer amino acid identity from 50% to 100% may be useful in describing the present invention, such as 51%, 52%, 53%, 54%, 55%, 56%, 57%, 58%, 59%, 60%, 61%, 62%, 63%, 64%, 65%, 66%, 67%, 68%, 69%, 70%, 71%, 72%, 73%, 74%, 75%, 76%, 77%, 78%, 79%, 80%, 81%, 82%, 83%, 84%, 85%, 86%, 87%, 88%, 89%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99%. Also, of interest is any full-length or partial complement of this isolated nucleotide fragment.

"Codon degeneracy" refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid fragment that encodes all or a substantial portion of the amino acid sequence encoding the instant euglenoid polypeptides as set forth in SEQ ID NO:13 and SEQ ID NO:14. The skilled artisan is well aware of the "codon-bias" exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

"Synthetic genes" can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell, where sequence information is available.

"Gene" refers to a nucleic acid fragment that expresses a specific protein, and that may refer to the coding region alone or may include regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence. "Native gene" refers to a gene as found in nature with its own regulatory sequences. "Chimeric gene" refers to any gene that is not a native gene, comprising regulatory and coding sequences that are not found together in nature. Accordingly, a chimeric gene may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. "Endogenous gene" refers to a native gene in its natural location in the genome of an organism. A "foreign" gene refers to a gene not normally found in the host organism, but that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, or chimeric genes. A "transgene" is a gene that has been introduced into the genome by a transformation procedure. A "codon-optimized gene" is a gene having its frequency of codon usage designed to mimic the frequency of preferred codon usage of the host cell.

"Coding sequence" refers to a DNA sequence that codes for a specific amino acid sequence. "Regulatory sequences" refer to nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of a coding sequence, and which influence the transcription, RNA processing or stability, or translation of the associated coding sequence. Regulatory sequences may include, but are not limited to: promoters, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

"Promoter" refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental conditions. Promoters that cause a gene to be expressed

at almost all stages of development, are commonly referred to as "constitutive promoters". It is further recognized that since in most cases the exact boundaries of regulatory sequences, especially at its 5' end, have not been completely defined, DNA fragments of some variation may have identical promoter activity.

A promoter sequence may consist of proximal and more distal upstream elements, the latter elements often referred to as enhancers and/or silencers. Accordingly, an "enhancer" is a DNA sequence that can stimulate promoter activity, and may be an innate element of the promoter or a heterologous element inserted to enhance the level or stage-specific activity of a promoter. A "silencer" is a DNA sequence that can repress promoter activity, and may be an innate element of the promoter or a heterologous element inserted to repress the level or stage-specific activity of a promoter.

"Translation leader sequence" refers to a polynucleotide sequence located between the transcription start site of a gene and the coding sequence. The translation leader sequence is present in the fully processed mRNA upstream of the translation start sequence. The translation leader sequence may affect processing of the primary transcript to mRNA, mRNA stability or translation efficiency. Examples of translation leader sequences have been described (Turner, R. and Foster, G. D., *Mol. Biotechnol.*, 3:225-236 (1995)).

The terms "3' non-coding sequences", "transcription terminator" and "termination sequences" refer to DNA sequences located downstream of a coding sequence. This includes polyadenylation recognition sequences and other sequences encoding regulatory signals capable of affecting mRNA processing or gene expression. The polyadenylation signal is usually characterized by affecting the addition of polyadenylic acid tracts to the 3' end of the mRNA precursor. The 3' region can influence the transcription, RNA processing or stability, or translation of the associated coding sequence.

"RNA transcript" refers to the product resulting from RNA polymerase-catalyzed transcription of a DNA sequence. When the RNA transcript is a perfect complementary copy of the DNA sequence, it is referred to as the primary transcript. A RNA transcript is referred to as the mature RNA when it is a RNA sequence derived from post-transcriptional processing of the primary transcript. "Messenger RNA" or "mRNA" refers to the RNA that is without introns and that can be translated

into protein by the cell. "cDNA" refers to a DNA that is complementary to, and synthesized from, a mRNA template using the enzyme reverse transcriptase. The cDNA can be single-stranded or converted into double-stranded form using the Klenow fragment of DNA polymerase I. "Sense" RNA refers to RNA transcript that includes the mRNA and can be translated into protein within a cell or *in vitro*. "Antisense RNA" refers to an RNA transcript that is complementary to all or part of a target primary transcript or mRNA, and that blocks the expression of a target gene (U.S. Patent 5,107,065). The complementarity of an antisense RNA may be with any part of the specific gene transcript, i.e., at the 5' non-coding sequence, 3' non-coding sequence, introns, or the coding sequence. "Functional RNA" refers to antisense RNA, ribozyme RNA, or other RNA that may not be translated but yet has an effect on cellular processes. The terms "complement" and "reverse complement" are used interchangeably herein with respect to mRNA transcripts, and are meant to define the antisense RNA of the message.

The term "operably linked" refers to the association of nucleic acid sequences on a single nucleic acid fragment so that the function of one is affected by the other. For example, a promoter is operably linked with a coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in a sense or antisense orientation.

The term "recombinant" refers to an artificial combination of two otherwise separated segments of sequence, e.g., by chemical synthesis or by the manipulation of isolated segments of nucleic acids by genetic engineering techniques.

The term "expression", as used herein, refers to the transcription and stable accumulation of sense (mRNA) or antisense RNA derived from the nucleic acid fragments of the invention. Expression may also refer to translation of mRNA into a protein (either precursor or mature).

"Mature" protein refers to a post-translationally processed polypeptide (i.e., one from which any pre- or propeptides present in the primary translation product have been removed). "Precursor" protein refers to the primary product of translation of mRNA (i.e., with pre- and propeptides still present). Pre- and propeptides may be but are not limited to intracellular localization signals.

The terms “plasmid” and “vector” refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of the cell, and usually in the form of circular double-stranded DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of introducing an expression cassette(s) into a cell.

The term “expression cassette” refers to a fragment of DNA comprising the coding sequence of a selected gene and regulatory sequences preceding (5' non-coding sequences) and following (3' non-coding sequences) the coding sequence that are required for expression of the selected gene product. Thus, an expression cassette is typically composed of: (1) a promoter sequence; (2) a coding sequence (i.e., ORF); and, (3) a 3' untranslated region (i.e., a terminator) that, in eukaryotes, usually contains a polyadenylation site. The expression cassette(s) is usually included within a vector, to facilitate cloning and transformation. Different expression cassettes can be transformed into different organisms including bacteria, yeast, plants and mammalian cells, as long as the correct regulatory sequences are used for each host.

A “recombinant DNA construct” (also referred to interchangeably herein as a “expression construct” and “construct”) comprises an artificial combination of nucleic acid fragments, e.g., regulatory and coding sequences that are not found together in nature. For example, a recombinant DNA construct may comprise regulatory sequences and coding sequences that are derived from different sources, or regulatory sequences and coding sequences derived from the same source, but arranged in a manner different than that found in nature. Such a construct may be used by itself or may be used in conjunction with a vector. If a vector is used, then the choice of vector is dependent upon the method that will be used to transform host cells as is well known to those skilled in the art. For example, a plasmid vector can be used. The skilled artisan is well aware of the genetic elements that must be present on the vector in order to successfully transform, select and propagate host cells comprising any of the isolated nucleic acid fragments of the invention. The skilled artisan will also recognize that different independent transformation events

will result in different levels and patterns of expression (Jones et al., *EMBO J.*, 4:2411-2418 (1985); De Almeida et al., *Mol. Gen. Genetics,* 218:78-86 (1989)), and thus that multiple events must be screened in order to obtain lines displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA, Northern analysis of mRNA expression, immunoblotting analysis of protein expression, or phenotypic analysis, among others.

The term “introduced” means providing a nucleic acid (e.g., expression cassette) or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. Introduced includes reference to stable or transient transformation methods, as well as sexually crossing. Thus, “introduced” in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct or expression cassette) into a cell, means “transfection” or “transformation” or “transduction” and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

As used herein, "transgenic" refers to a cell which comprises within its genome a heterologous polynucleotide. Preferably, the heterologous polynucleotide is stably integrated within the genome such that the polynucleotide is passed on to successive generations. The heterologous polynucleotide may be integrated into the genome alone or as part of an expression cassette. Transgenic is used herein to include any cell or cell line, the genotype of which has been altered by the presence of heterologous nucleic acids including those transgenics initially so altered as well as those created by mating from the initial transgenic with different mating types. The term "transgenic" as used herein does not encompass the alteration of the genome (chromosomal or extra-chromosomal) by naturally occurring events such as random cross-fertilization, non-recombinant viral infection, non-recombinant bacterial transformation, non-recombinant transposition, or spontaneous mutation.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described more fully in Sambrook, J., Fritsch, E.F. and Maniatis, T. Molecular Cloning: A Laboratory Manual; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989); by Silhavy, T. J., Bennan, M. L. and Enquist, L. W., Experiments with Gene Fusions, Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and by Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience, Hoboken, NJ (1987). Transformation methods are well known to those skilled in the art and are described *infra*.An Overview: Microbial Biosynthesis Of Fatty Acids And Triacylglycerols

In general, lipid accumulation in oleaginous microorganisms is triggered in response to the overall carbon to nitrogen ratio present in the growth medium. This process, leading to the *de novo* synthesis of free palmitate (16:0) in oleaginous microorganisms, is described in detail in U.S. Patent 7,238,482. Palmitate is the precursor of longer-chain saturated and unsaturated fatty acid derivates, which are formed through the action of elongases and desaturases (FIG. 1).

TAGs (the primary storage unit for fatty acids) are formed by a series of reactions that involve: (1) the esterification of one molecule of acyl-CoA to glycerol-3-phosphate via an acyltransferase to produce lysophosphatidic acid; (2) the esterification of a second molecule of acyl-CoA via an acyltransferase to yield 1,2-diacylglycerol phosphate (commonly identified as phosphatidic acid); (3) removal of a phosphate by phosphatidic acid phosphatase to yield 1,2-diacylglycerol (DAG); and, (4) the addition of a third fatty acid by the action of an acyltransferase to form TAG. A wide spectrum of fatty acids can be incorporated into TAGs, including saturated and unsaturated fatty acids and short-chain and long-chain fatty acids.

Biosynthesis Of Omega Fatty Acids

The metabolic process wherein oleic acid is converted to ω-3/ω-6 fatty acids involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the addition of double bonds. This requires a series of special desaturation and elongation enzymes present in the endoplasmic reticulim membrane. However, as seen in FIG. 1 and as described below, there are often multiple alternate pathways for production of a specific ω-3/ω-6 fatty acid.

Specifically, all pathways require the initial conversion of oleic acid to LA, the first of the ω-6 fatty acids, by a Δ12 desaturase. Then, using the "Δ9 elongase/Δ8 desaturase pathway" and LA as substrate, long chain ω-6 fatty acids are formed as follows: (1) LA is converted to EDA by a Δ9 elongase; (2) EDA is converted to DGLA by a Δ8 desaturase; (3) DGLA is converted to ARA by a Δ5 desaturase; (4) ARA is converted to DTA by a $C_{20/22}$ elongase; and, (5) DTA is converted to DPAn-6 by a Δ4 desaturase. Alternatively, the "Δ9 elongase/Δ8 desaturase pathway" can use ALA as substrate to produce long chain ω-3 fatty acids as follows: (1) LA is converted to ALA, the first of the ω-3 fatty acids, by a Δ15 desaturase; (2) ALA is converted to ETrA by a Δ9 elongase; (3) ETrA is converted to ETA by a Δ8 desaturase; (4) ETA is converted to EPA by a Δ5 desaturase; (5) EPA is converted to DPA by a $C_{20/22}$ elongase; and, (6) DPA is converted to DHA by a Δ4 desaturase. Optionally, ω-6 fatty acids may be converted to ω-3 fatty acids; for example, ALA is produced from LA by Δ15 desaturase activity; ETA and EPA are produced from DGLA and ARA, respectively, by Δ17 desaturase activity.

Alternate pathways for the biosynthesis of ω-3/ω-6 fatty acids utilize a Δ6 desaturase and $C_{18/20}$ elongase (i.e., the "Δ6 desaturase/Δ6 elongase pathway"). More specifically, LA and ALA may be converted to GLA and STA, respectively, by a Δ6 desaturase; then, a $C_{18/20}$ elongase converts GLA to DGLA and/or STA to ETA. Downstream PUFAs are subsequently formed as described above.

It is contemplated that the particular functionalities required to be introduced into a specific host organism for production of ω-3/ω-6 fatty acids will depend on the host cell (and its native PUFA profile and/or desaturase/elongase profile), the availability of substrate, and the desired end product(s). For example, expression of the Δ9 elongase/Δ8 desaturase pathway may be preferred in some embodiments, as opposed to expression of the Δ6 desaturase/Δ6 elongase pathway, since PUFAs produced via the former pathway are devoid of GLA and/or STA.

One skilled in the art will be able to identify various candidate genes encoding each of the enzymes desired for ω-3/ω-6 fatty acid biosynthesis. Useful desaturase and elongase sequences may be derived from any source, e.g., isolated from a natural source (from bacteria, algae, fungi, plants, animals, etc.), produced via a semi-synthetic route or synthesized *de novo*. Although the particular source of the

desaturase and elongase genes introduced into the host is not critical, considerations for choosing a specific polypeptide having desaturase or elongase activity include: (1) the substrate specificity of the polypeptide; (2) whether the polypeptide or a component thereof is a rate-limiting enzyme; (3) whether the desaturase or elongase is essential for synthesis of a desired PUFA; (4) co-factors required by the polypeptide; and/or, (5) whether the polypeptide was modified after its production (e.g., by a kinase or a prenyltransferase). The expressed polypeptide preferably has parameters compatible with the biochemical environment of its location in the host cell (see U.S. Patent 7,238,482 for additional details).

In additional embodiments, it will also be useful to consider the conversion efficiency of each particular desaturase and/or elongase. More specifically, since each enzyme rarely functions with 100% efficiency to convert substrate to product, the final lipid profile of unpurified oils produced in a host cell will typically be a mixture of various PUFAs consisting of the desired ω-3/ω-6 fatty acid, as well as various upstream intermediary PUFAs. Thus, each enzyme's conversion efficiency is also a variable to consider, when optimizing biosynthesis of a desired fatty acid.

With each of the considerations above in mind, candidate genes having the appropriate desaturase and elongase activities (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ5 desaturases, Δ17 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ8 desaturases, Δ4 desaturases and $C_{20/22}$ elongases) can be identified according to publicly available literature (e.g., GenBank), the patent literature, and experimental analysis of organisms having the ability to produce PUFAs. These genes will be suitable for introduction into a specific host organism, to enable or enhance the organism's synthesis of PUFAs.

Sequence Identification Of Novel Δ9 Elongases

In the present invention, nucleotide sequences encoding Δ9 elongases have been isolated from *Euglena anabaena,* as summarized below in Table 3.

Table 3

Summary Of *Euglena anabaena* Δ9 Elongases

Abbreviation	Nucleotide SEQ ID NO	Amino Acid SEQ ID NO
EaD9Elo1	11	13

EaD9Elo2	12	14
EaD9eS	26	27

*Note: SEQ ID NO:27 is identical in sequence to SEQ ID NO:13.

Thus, the present invention concerns an isolated polynucleotide comprising:

(a) a nucleotide sequence encoding a polypeptide having Δ9 elongase activity, wherein the polypeptide has at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14;

(b) a nucleotide sequence encoding a polypeptide having Δ9 elongase activity, wherein the nucleotide sequence has at least 80% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:26; or,

(c) a complement of the nucleotide sequence of (a) or (b), wherein the complement and the nucleotide sequence consist of the same number of nucleotides and are 100% complementary.

In still another aspect, this invention concerns an isolated polynucleotide comprising a nucleotide sequence encoding a polypeptide having Δ9 elongase activity, wherein the nucleotide sequence has at least 90% sequence identity, based on the BLASTN method of alignment, when compared to a nucleotide sequence as set forth in SEQ ID NO:11, SEQ ID NO:12 or SEQ ID NO:26.

More preferred amino acid fragments that are at least about 80%-90% identical are particularly suitable and those sequences that are at least about 90%-95% identical are most preferred. Similarly, preferred Δ9 elongase encoding nucleic acid sequences corresponding to the instant ORFs are those encoding active proteins and which are at least about 80%-90% identical; those sequences that are at least about 90%-95% identical are most preferred.

In alternate embodiments, the instant EaD9Elo1 and/or EaD9Elo2 sequences can be codon-optimized for expression in a particular host organism. As is well known in the art, this can be a useful means to further optimize the expression of the enzyme in the alternate host, since use of host-preferred codons can substantially enhance the expression of the foreign gene encoding the polypeptide. In general, host-preferred codons can be determined within a

particular host species of interest by examining codon usage in proteins (preferably those expressed in the largest amount) and determining which codons are used with highest frequency. Then, the coding sequence for a polypeptide of interest having e.g., elongase activity can be synthesized in whole or in part using the codons preferred in the host species. All (or portions) of the DNA also can be synthesized to remove any destabilizing sequences or regions of secondary structure that would be present in the transcribed mRNA. All (or portions) of the DNA also can be synthesized to alter the base composition to one more preferable in the desired host cell.

In one embodiment of the invention herein, EaD9Elo1 (SEQ ID NO:11) was codon-optimized for expression in *Yarrowia lipolytica.* This was possible based on previous determination of the *Y. lipolytica* codon usage profile, identification of those codons that were preferred, and determination of the consensus sequence around the 'ATG' initiation codon (see U.S. Patent 7,238,482 and U.S. Patent 7,125,672). The resultant synthetic gene is referred to as EaD9ES (SEQ ID NO:26). The protein sequence encoded by the codon-optimized Δ9 elongase gene (i.e., SEQ ID NO:27) is identical to that of the wildtype protein sequence (i.e., SEQ ID NO:13). Similar techniques could be utilized to produce a synthetic Δ9 elongase derived from EaD9Elo2 (SEQ ID NO:12) for expression in *Y. lipolytica*.

One skilled in the art would be able to use the teachings herein to create various other codon-optimized Δ9 elongase proteins suitable for optimal expression in alternate hosts (i.e., other than *Yarrowia lipolytica*), based on the wildtype EaD9Elo1 and/or EaD9Elo2 sequences. Accordingly, the instant invention relates to any codon-optimized Δ9 elongase protein that is derived from the wildtype nucleotide sequences of EaD9Elo1 (SEQ ID NO:11) or EaD9Elo2 (SEQ ID NO:12). This includes, but is not limited to, the nucleotide sequence set forth in SEQ ID NO:26, which encodes a synthetic Δ9 elongase protein (i.e., EaD9eS) that was codon-optimized for expression in *Yarrowia lipolytica.* In alternate embodiments, it may be desirable to modify a portion of the codons encoding EaD9Elo1 and/or EaD9Elo2 to enhance expression of the gene in a host organism including, but not limited to, a plant or plant part, algae, bacteria, alternate yeast, euglenoid, stramenopiles or fungi.

Identification And Isolation Of Homologs

Any of the instant elongase sequences (i.e., EaD9Elo1, EaD9Elo2, or EaD9eS) or portions thereof may be used to search for Δ9 elongase homologs in the same or other bacterial, algal, fungal, euglenoid or plant species using sequence analysis software. In general, such computer software matches similar sequences by assigning degrees of homology to various substitutions, deletions, and other modifications.

Alternatively, any of the instant elongase sequences or portions thereof may also be employed as hybridization reagents for the identification of Δ9 elongase homologs. The basic components of a nucleic acid hybridization test include a probe, a sample suspected of containing the gene or gene fragment of interest and a specific hybridization method. Probes of the present invention are typically single-stranded nucleic acid sequences that are complementary to the nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected. Although the probe length can vary from 5 bases to tens of thousands of bases, typically a probe length of about 15 bases to about 30 bases is suitable. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base.

Hybridization methods are well defined. Typically the probe and sample must be mixed under conditions that will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be in contact for a long enough time that any possible hybridization between the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added (e.g., guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium

iodide, cesium trifluoroacetate). If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

Various hybridization solutions can be employed. Typically, these comprise from about 20 to 60% volume, preferably 30%, of a polar organic solvent. A common hybridization solution employs about 30-50% v/v formamide, about 0.15 to 1 M sodium chloride, about 0.05 to 0.1 M buffers (e.g., sodium citrate, Tris-HCl, PIPES or HEPES (pH range about 6-9)), about 0.05 to 0.2% detergent (e.g., sodium dodecylsulfate), or between 0.5-20 mM EDTA, FICOLL (Pharmacia Inc.) (about 300-500 kdal), polyvinylpyrrolidone (about 250-500 kdal), and serum albumin. Also included in the typical hybridization solution will be unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA (e.g., calf thymus or salmon sperm DNA, or yeast RNA), and optionally from about 0.5 to 2% wt/vol glycine. Other additives may also be included, such as volume exclusion agents that include a variety of polar water-soluble or swellable agents (e.g., polyethylene glycol), anionic polymers (e.g., polyacrylate or polymethylacrylate) and anionic saccharidic polymers (e.g., dextran sulfate).

Nucleic acid hybridization is adaptable to a variety of assay formats. One of the most suitable is the sandwich assay format. The sandwich assay is particularly adaptable to hybridization under non-denaturing conditions. A primary component of a sandwich-type assay is a solid support. The solid support has adsorbed to it or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the sequence.

In additional embodiments, any of the Δ9 elongase nucleic acid fragments described herein (or any homologs identified thereof) may be used to isolate genes encoding homologous proteins from the same or other bacterial, algal, fungal, euglenoid or plant species. Isolation of homologous genes using sequence-dependent protocols is well known in the art. Examples of sequence-dependent protocols include, but are not limited to: (1) methods of nucleic acid hybridization; (2) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Patent 4,683,202; ligase chain reaction (LCR), Tabor et al., *Proc. Acad. Sci. U.S.A.*, 82:1074 (1985); or strand displacement amplification (SDA),

Walker et al., *Proc. Natl. Acad. Sci. U.S.A.*, 89:392 (1992)]; and, (3) methods of library construction and screening by complementation.

For example, genes encoding similar proteins or polypeptides to the Δ9 elongases described herein could be isolated directly by using all or a portion of the instant nucleic acid fragments as DNA hybridization probes to screen libraries from e.g., any desired yeast or fungus using methodology well known to those skilled in the art (wherein those organisms producing EDA and/or ETrA would be preferred). Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis, *supra*). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan (e.g., random primers DNA labeling, nick translation or end-labeling techniques), or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of (or full-length of) the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full-length DNA fragments under conditions of appropriate stringency.

Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending on the desired test conditions, the sequences of the primers should be designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art (Thein and Wallace, "The use of oligonucleotide as specific hybridization probes in the Diagnosis of Genetic Disorders", in *Human Genetic Diseases: A Practical Approach*, K. E. Davis Ed., (1986) pp 33-50, IRL: Herndon, VA; and Rychlik, W., In Methods in Molecular Biology, White, B. A. Ed., (1993) Vol. 15, pp 31-39, PCR Protocols: Current Methods and Applications. Humania: Totowa, NJ).

Generally two short segments of the instant sequences may be used in PCR protocols to amplify longer nucleic acid fragments encoding homologous genes from DNA or RNA. PCR may also be performed on a library of cloned nucleic acid fragments wherein the sequence of one primer is derived from the instant nucleic acid fragments, and the sequence of the other primer takes advantage of the

presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding eukaryotic genes.

Alternatively, the second primer sequence may be based upon sequences derived from the cloning vector. For example, the skilled artisan can follow the RACE protocol (Frohman et al., *Proc. Acad. Sci. U.S.A.*, 85:8998 (1988)) to generate cDNAs by using PCR to amplify copies of the region between a single point in the transcript and the 3' or 5' end. Primers oriented in the 3' and 5' directions can be designed from the instant sequences. Using commercially available 3' RACE or 5' RACE systems (Gibco/BRL, Gaithersburg, MD), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., *Proc. Acad. Sci. U.S.A.*, 86:5673 (1989); Loh et al., *Science,* 243:217 (1989)).

In other embodiments, any of the Δ9 elongase nucleic acid fragments described herein (or any homologs identified thereof) may be used for creation of new and/or improved fatty acid elongases. As is well known in the art, *in vitro* mutagenesis and selection, chemical mutagenesis, "gene shuffling" methods or other means can be employed to obtain mutations of naturally occurring elongase genes (wherein such mutations may include deletions, insertions and point mutations, or combinations thereof). This would permit production of a polypeptide having elongase activity, respectively, *in vivo* with more desirable physical and kinetic parameters for function in the host cell such as a longer half-life or a higher rate of production of a desired PUFA. Or, if desired, the regions of a polypeptide of interest (i.e., a Δ9 elongase) important for enzymatic activity can be determined through routine mutagenesis, expression of the resulting mutant polypeptides and determination of their activities. An overview of these techniques is described in U.S. Patent 7,238,482. All such mutant proteins and nucleotide sequences encoding them that are derived from EaD9Elo1, EaD9Elo2 and EaD9eS are within the scope of the present invention.

Alternatively, improved fatty acids may be synthesized by domain swapping, wherein a functional domain from any of the Δ9 elongase nucleic acid fragments described herein are exchanged with a functional domain in an alternate elongase gene to thereby result in a novel protein. As used herein, "domain" or "functional domain" refer to nucleic acid sequence(s) that are capable of eliciting a biological response in microbes.

Methods For Production Of Various Omega-3 And/Or Omega-6 Fatty Acids

It is expected that introduction of chimeric genes encoding the Δ9 elongases described herein (i.e., EaD9Elo1, EaD9Elo2, EaD9eS or other mutant enzymes, codon-optimized enzymes or homologs thereof), under the control of the appropriate promoters will result in increased production of EDA and/or ETrA in the transformed host organism, respectively. As such, the present invention encompasses a method for the direct production of PUFAs comprising exposing a fatty acid substrate (i.e., LA and/or ALA) to the elongase enzymes described herein (e.g., EaD9Elo1, EaD9Elo2 or EaD9eS), such that the substrate is converted to the desired fatty acid product (i.e., EDA and/or ETrA, respectively).

More specifically, it is an object of the present invention to provide a method for the production of EDA in a microbial host cell (e.g., yeast, algae, bacteria, euglenoids, stramenopiles and fungi), wherein the microbial host cell comprises:

(a) a recombinant nucleotide molecule encoding a Δ9 elongase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14; and,

(b) a source of LA;

wherein the microbial host cell is grown under conditions such that the nucleic acid fragment encoding the Δ9 elongase is expressed and the LA is converted to EDA, and wherein the EDA is optionally recovered.

In alternate embodiments of the present invention, the Δ9 elongase may be used for the conversion of ALA to ETrA. Accordingly the invention provides a method for the production of ETrA, wherein the microbial host cell comprises:

(a) a recombinant nucleotide molecule encoding a Δ9 elongase polypeptide having at least 80% amino acid identity, based on the Clustal V method of alignment, when compared to a polypeptide having an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14; and,

(b) a source of ALA;

wherein the microbial host cell is grown under conditions such that the nucleic acid fragment encoding the Δ9 elongase is expressed and the ALA is converted to ETrA, and wherein the ETrA is optionally recovered.

Alternatively, each Δ9 elongase gene and its corresponding enzyme product described herein can be used indirectly for the production of various ω-6 and ω-3 PUFAs (see FIG. 1 and U.S. Patent 7,238,482). Indirect production of ω-3/ω-6 PUFAs occurs wherein the fatty acid substrate is converted indirectly into the desired fatty acid product, via means of an intermediate step(s) or pathway intermediate(s). Thus, it is contemplated that the Δ9 elongases described herein (i.e., EaD9Elo1, EaD9Elo2, EaD9eS or other mutant enzymes, codon-optimized enzymes or homologs thereof) may be expressed in conjunction with additional genes encoding enzymes of the PUFA biosynthetic pathway (e.g., Δ6 desaturases, $C_{18/20}$ elongases, Δ17 desaturases, Δ8 desaturases, Δ15 desaturases, Δ9 desaturases, Δ12 desaturases, $C_{14/16}$ elongases, $C_{16/18}$ elongases, Δ9 elongases, Δ5 desaturases, Δ4 desaturases, $C_{20/22}$ elongases) to result in higher levels of production of longer-chain ω-3/ω-6 fatty acids (e.g., ARA, EPA, DTA, DPAn-6, DPA and/or DHA).

In preferred embodiments, the Δ9 elongases of the present invention will minimally be expressed in conjunction with a Δ8 desaturase (e.g., from *Euglena gracilis* [Wallis et al., *Arch. Biochem. and Biophys.,* 365(2):307-316 (May 1999); PCT Publication No. WO 2000/34439; U.S. Patent No. 6,825,017; PCT Publication No. WO 2004/057001; PCT Publication No. WO 2006/012325; U.S. Patent No. 7,256,033; U.S. Patent Application No. 11/635258]; from *Acanthamoeba castellanii* [Sayanova et al., *FEBS Lett.,* 580:1946-1952 (2006)]; from *Pavlova salina* [PCT Publication No. WO 2005/103253]; from *Pavlova lutheri* [PCT Publication No. WO 2007/127381]; from *Tetruetreptia pomquetensis* CCMP1491 [U.S. Patent Application No. 11/876115]; from *Eutreptiella sp.* CCMP389 [U.S. Patent Application No. 11/876115]; from *Eutreptiella cf_gymnastica* CCMP1594 [U.S. Patent Application No. 11/876115; and, from *Euglena anabaena* [described in co-pending U.S. Patent Applications No. 12/099799 and No. 12/099811]). However, the particular genes included within a particular expression cassette will depend on the host cell (and its PUFA profile and/or desaturase/elongase profile), the availability of substrate and the desired end product(s).

In alternative embodiments, it may be useful to disrupt a host organism's native Δ9 elongase, based on the complete sequences described herein, the complement of those complete sequences, substantial portions of those sequences,

codon-optimized elongases derived therefrom and those sequences that are substantially homologous thereto.

Microbial Expression Systems, Cassettes And Vectors

The Δ9 elongase genes and gene products described herein (i.e., EaD9Elo1, EaD9Elo2, EaD9eS or other mutant enzymes, codon-optimized enzymes or homologs thereof) may be expressed in heterologous microbial host cells, particularly in the cells of oleaginous yeasts (e.g., *Yarrowia lipolytica*).

Microbial expression systems and expression vectors containing regulatory sequences that direct high level expression of foreign proteins are well known to those skilled in the art. Any of these could be used to construct chimeric genes for production of any of the gene products of the instant sequences. These chimeric genes could then be introduced into appropriate microorganisms via transformation to provide high-level expression of the encoded enzymes.

Vectors (e.g., constructs, plasmids) and DNA expression cassettes useful for the transformation of suitable microbial host cells are well known in the art. The specific choice of sequences present in the construct is dependent upon the desired expression products (*supra*), the nature of the host cell and the proposed means of separating transformed cells versus non-transformed cells. Typically, however, the vector contains at least one expression cassette, a selectable marker and sequences allowing autonomous replication or chromosomal integration. Suitable expression cassettes comprise a region 5' of the gene that controls transcription (e.g., a promoter), the gene coding sequence, and a region 3' of the DNA fragment that controls transcriptional termination (i.e., a terminator). It is most preferred when both control regions are derived from genes from the transformed microbial host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

Transcriptional control regions (also initiation control regions or promoters) which are useful to drive expression of the instant Δ9 elongase ORFs in the desired microbial host cell are numerous and familiar to those skilled in the art. Virtually any promoter (i.e., native, synthetic, or chimeric) capable of directing expression of these genes in the selected host cell is suitable for the present invention, although transcriptional and translational regions from the host species are particularly useful. Expression in a microbial host cell can be accomplished in an induced or

constitutive fashion. Induced expression can be accomplished by inducing the activity of a regulatable promoter operably linked to the gene of interest, while constitutive expression can be achieved by the use of a constitutive promoter operably linked to the gene of interest. As an example, when the host cell is yeast, transcriptional and translational regions functional in yeast cells are provided, particularly from the host species (e.g., see Patent Publication No. US-2006-0115881-A1 for preferred transcriptional initiation regulatory regions for use in *Yarrowia lipolytica*). Any one of a number of regulatory sequences can be used, depending upon whether constitutive or induced transcription is desired, the efficiency of the promoter in expressing the ORF of interest, the ease of construction and the like.

Nucleotide sequences surrounding the translational initiation codon 'ATG' have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence to obtain optimal gene expression. For expression in yeast, this can be done by site-directed mutagenesis of an inefficiently expressed gene by fusing it in-frame to an endogenous yeast gene, preferably a highly expressed gene. Alternatively, one can determine the consensus translation initiation sequence in the host and engineer this sequence into heterologous genes for their optimal expression in the host of interest.

The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and function satisfactorily in a variety of hosts (when utilized both in the same and different genera and species from where they were derived). The termination region usually is selected more as a matter of convenience rather than because of any particular property. Termination control regions may also be derived from various genes native to the preferred hosts. In alternate embodiments, the 3'-region can also be synthetic, as one of skill in the art can utilize available information to design and synthesize a 3'-region sequence that functions as a transcription terminator. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

As one of skill in the art is aware, merely inserting a gene into a cloning vector does not ensure that it will be successfully expressed at the level needed. In response to the need for a high expression rate, many specialized expression vectors have been created by manipulating a number of different genetic elements that control aspects of transcription, translation, protein stability, oxygen limitation and secretion from the microbial host cell. More specifically, some of the molecular features that have been manipulated to control gene expression include: the nature of the relevant transcriptional promoter and terminator sequences; the number of copies of the cloned gene (wherein additional copies may be cloned within a single expression construct and/or additional copies may be introduced into the host cell by increasing the plasmid copy number or by multiple integration of the cloned gene into the genome); whether the gene is plasmid-borne or integrated into the genome of the host cell; the final cellular location of the synthesized foreign protein; the efficiency of translation and correct folding of the protein in the host organism; the intrinsic stability of the mRNA and protein of the cloned gene within the host cell; and, the codon usage within the cloned gene, such that its frequency approaches the frequency of preferred codon usage of the host cell. Each of these types of modifications are encompassed in the present invention, as means to further optimize expression of the Δ9 elongase described herein.

Transformation Of Microbial Host Cells

Once a DNA cassette that is suitable for expression in an appropriate microbial host cell has been obtained (e.g., a chimeric gene comprising a promoter, ORF and terminator), it is placed in a plasmid vector capable of autonomous replication in a host cell, or it is directly integrated into the genome of the host cell. Integration of expression cassettes can occur randomly within the host genome or can be targeted through the use of constructs containing regions of homology with the host genome sufficient to target recombination within the host locus. Where constructs are targeted to an endogenous locus, all or some of the transcriptional and translational regulatory regions can be provided by the endogenous locus.

Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of selection and should lack homology to the other construct(s) to maintain stable expression and prevent reassortment of elements among constructs. Judicious choice of regulatory regions,

selection means and method of propagation of the introduced construct(s) can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

Constructs comprising the gene(s) of interest may be introduced into a microbial host cell by any standard technique. These techniques include transformation (e.g., lithium acetate transformation [*Methods in Enzymology*, 194:186-187 (1991)]), protoplast transformation, bolistic impact, electroporation, microinjection, or any other method that introduces the gene(s) of interest into the host cell.

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence (e.g., an expression cassette) will be referred to as "transformed", "transformant" or "recombinant" herein. Thus, the term "transformed" and "recombinant" are used interchangeably herein. The transformed host will have at least one copy of the expression construct and may have two or more, depending upon whether the expression cassette is integrated into the genome or is present on an extrachromosomal element having multiple copy numbers.

The transformed host cell can be identified by various selection techniques, as described in U.S. Patent 7,238,482, U.S. Patent 7,259,255 and PCT Publication No. WO 2006/052870.

Following transformation, substrates suitable for the instant Δ9 elongase (and, optionally other PUFA enzymes that are co-expressed within the host cell) may be produced by the host either naturally or transgenically, or they may be provided exogenously.

Preferred Microbial Hosts For Recombinant Expression

Microbial host cells for expression of the instant genes and nucleic acid fragments may include hosts that grow on a variety of feedstocks, including simple or complex carbohydrates, fatty acids, organic acids, oils, glycerol and alcohols, and/or hydrocarbons over a wide range of temperature and pH values. Based on the needs of the Applicants' Assignee, the genes described in the instant invention have been expressed in an oleaginous yeast (and in particular *Yarrowia lipolytica*); however, it is contemplated that because transcription, translation and the protein biosynthetic apparatus are highly conserved, any bacteria, yeast, algae, euglenoid,

stramenopiles and/or fungus will be a suitable microbial host for expression of the present nucleic acid fragments.

Preferred microbial hosts, however, are oleaginous organisms, such as oleaginous yeasts. These organisms are naturally capable of oil synthesis and accumulation, wherein the oil can comprise greater than about 25% of the cellular dry weight, more preferably greater than about 30% of the cellular dry weight, and most preferably greater than about 40% of the cellular dry weight. Genera typically identified as oleaginous yeast include, but are not limited to: *Yarrowia, Candida, Rhodotorula, Rhodosporidium*, *Cryptococcus, Trichosporon* and *Lipomyces*. More specifically, illustrative oil-synthesizing yeasts include: *Rhodosporidium toruloides, Lipomyces starkeyii, L. lipoferus*, *Candida revkaufi, C. pulcherrima, C. tropicalis, C. utilis, Trichosporon pullans, T. cutaneum, Rhodotorula glutinus, R. graminis,* and *Yarrowia lipolytica* (formerly classified as *Candida lipolytica*). In alternate embodiments, oil biosynthesis may be genetically engineered such that the microbial host cell (e.g., a yeast) can produce more than 25% oil of the cellular dry weight, and thereby be considered oleaginous.

Most preferred is the oleaginous yeast *Yarrowia lipolytica;* and, in a further embodiment, most preferred are the *Y. lipolytica* strains designated as ATCC #20362, ATCC #8862, ATCC #18944, ATCC #76982 and/or LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.*, 82(1):43-9 (2002)).

Historically, various strains of *Y. lipolytica* have been used for the manufacture and production of: isocitrate lyase; lipases; polyhydroxyalkanoates; citric acid; erythritol; 2-oxoglutaric acid; γ-decalactone; γ-dodecalatone; and pyruvic acid. Specific teachings applicable for transformation of oleaginous yeasts (i.e., *Yarrowia lipolytica*) include U.S. Patent 4,880,741 and U.S. Patent 5,071,764 and Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)). Specific teachings applicable for engineering ARA, EPA and DHA production in *Y. lipolytica* are provided in U.S. Patent Application No. 11/264784, U.S. Patent Application No. 11/265761, and U.S. Patent Application No. 11/264737, respectively. The preferred method of expressing genes in this yeast is by integration of linear DNA into the genome of the host; and, integration into multiple locations within the genome can be particularly useful when high level expression of genes are desired [e.g., in the *Ura3* locus (GenBank Accession No. AJ306421), the *Leu2* gene locus (GenBank

Accession No. AF260230), the *Lys5* gene locus (GenBank Accession No. M34929), the *Aco2* gene locus (GenBank Accession No. AJ001300), the *Pox3* gene locus (Pox3: GenBank Accession No. XP_503244; or, Aco3: GenBank Accession No. AJ001301), the Δ12 desaturase gene locus (U.S. Patent 7,214,491), the *Lip1* gene locus (GenBank Accession No. Z50020), the *Lip2* gene locus (GenBank Accession No. AJ012632), the *SCP2* gene locus (GenBank Accession No. AJ431362), and/or the Pex10 gene locus (GenBank Accession No. CAG81606)].

Preferred selection methods for use in *Yarrowia lipolytica* are resistance to kanamycin, hygromycin and the amino glycoside G418, as well as ability to grow on media lacking uracil, leucine, lysine, tryptophan or histidine. In alternate embodiments, 5-fluoroorotic acid (5-fluorouracil-6-carboxylic acid monohydrate; "5-FOA") is used for selection of yeast *Ura*$^-$ mutants. The compound is toxic to yeast cells that possess a functioning URA3 gene encoding orotidine 5'-monophosphate decarboxylase (OMP decarboxylase); thus, based on this toxicity, 5-FOA is especially useful for the selection and identification of *Ura*$^-$ mutant yeast strains (Bartel, P.L. and Fields, S., Yeast 2-Hybrid System, Oxford University: New York, v. 7, pp 109-147, 1997; see also PCT Publication No. WO 2006/052870 for 5-FOA use in *Yarrowia*).

An alternate preferred selection method for use in *Yarrowia* relies on a dominant, non-antibiotic marker for *Yarrowia lipolytica* based on sulfonylurea (chlorimuron ethyl; E. I. duPont de Nemours & Co., Inc., Wilmington, DE) resistance. More specifically, the marker gene is a native acetohydroxyacid synthase (AHAS or acetolactate synthase; E.C. 4.1.3.18) that has a single amino acid change (W497L) that confers sulfonyl urea herbicide resistance (PCT Publication No. WO 2006/052870). AHAS is the first common enzyme in the pathway for the biosynthesis of branched-chain amino acids (i.e., valine, leucine, isoleucine) and it is the target of the sulfonylurea and imidazolinone herbicides.

Other preferred microbial hosts include oleaginous bacteria, algae, euglenoids, stramenopiles and other fungi; and, within this broad group of microbial hosts, of particular interest are microorganisms that synthesize ω-3/ω-6 fatty acids (or those that can be genetically engineered for this purpose [e.g., other yeast such as *Saccharomyces cerevisiae*]). Thus, for example, transformation of *Mortierella alpina* (which is commercially used for production of ARA) with any of the present

Δ9 elongase genes under the control of inducible or regulated promoters could yield a transformant organism capable of synthesizing increased quantities of EDA; this could be converted to increased quantities of DGLA if a Δ8 desaturase gene was co-expressed. The method of transformation of *M. alpina* is described by Mackenzie et al. (*Appl. Environ. Microbiol.,* 66:4655 (2000)). Similarly, methods for transformation of Thraustochytriales microorganisms (e.g., *Thraustochytrium, Schizochytrium*) are disclosed in U.S. 7,001,772.

Irrespective of the host selected for expression of the Δ9 elongases described herein, it may be necessary to screen multiple transformants to obtain a strain displaying the desired expression level and pattern. Such screening may be accomplished by Southern analysis of DNA blots (Southern, *J. Mol. Biol.,* 98:503 (1975)), Northern analysis of mRNA expression (Kroczek, *J. Chromatogr. Biomed. Appl.*, 618(1-2):133-145 (1993)), Western and/or Elisa analyses of protein expression, phenotypic analysis or GC analysis of the PUFA products.

Based on the teachings described above, in one embodiment this invention is drawn to a method of producing either EDA or ETrA, respectively, comprising:

(a) providing an oleaginous yeast (e.g., *Yarrowia lipolytica*) comprising:
  (i) a first recombinant nucleotide molecule encoding a Δ9 elongase polypeptide, operably linked to at least one regulatory sequence; and,
  (ii) a source of elongase substrate consisting of LA and/or ALA, respectively; and,

(b) growing the yeast of step (a) in the presence of a suitable fermentable carbon source wherein the gene encoding the Δ9 elongase polypeptide is expressed and LA is converted to EDA and/or ALA is converted to ETrA, respectively; and,

(c) optionally recovering the EDA and/or ETrA, respectively, of step (b).

Substrate feeding may be required.

The nucleotide sequence of the gene encoding a Δ9 elongase may be selected from the group consisting of SEQ ID NO:11 and SEQ ID NO:12. In alternate embodiments, the nucleotide sequence of the gene encoding a Δ9 elongase polypeptide is set forth in SEQ ID NO:26 (wherein at least 98 codons have been optimized for expression in *Yarrowia* relative to SEQ ID NO:11).

Since naturally produced PUFAs in oleaginous yeast are limited to 18:2 fatty acids (i.e., LA), and less commonly, 18:3 fatty acids (i.e., ALA), the oleaginous yeast will be genetically engineered to express multiple enzymes necessary for long-chain PUFA biosynthesis (thereby enabling production of e.g., ARA, EPA, DPA and DHA), in addition to the Δ9 elongases described herein.

Specifically, in one embodiment this invention concerns an oleaginous yeast comprising:

(a) a first recombinant DNA construct comprising an isolated polynucleotide encoding a Δ9 elongase polypeptide, operably linked to at least one regulatory sequence; and,

(b) at least one additional recombinant DNA construct comprising an isolated polynucleotide, operably linked to at least one regulatory sequence, encoding a polypeptide selected from the group consisting of: a Δ4 desaturase, a Δ5 desaturase, Δ6 desaturase, a Δ9 desaturase, a Δ12 desaturase, a Δ15 desaturase, a Δ17 desaturase, a Δ8 desaturase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase and a $C_{20/22}$ elongase.

In particularly preferred embodiments, the at least one additional recombinant DNA construct encodes a polypeptide having Δ8 desaturase activity.

Metabolic Engineering Of Omega-3 And/Or Omega-6 Fatty Acid Biosynthesis In Microbes

Knowledge of the sequences of the present Δ9 elongases will be useful for manipulating ω-3 and/or ω-6 fatty acid biosynthesis in various host cells. Methods for manipulating biochemical pathways are well known to those skilled in the art; and, it is expected that numerous manipulations will be possible to maximize ω-3 and/or ω-6 fatty acid biosynthesis in oleaginous yeasts, and particularly, in *Yarrowia lipolytica*. This manipulation may require metabolic engineering directly within the PUFA biosynthetic pathway or additional manipulation of pathways that contribute carbon to the PUFA biosynthetic pathway. Methods useful for up-regulating desirable biochemical pathways and down-regulating undesirable biochemical pathways are well known to those skilled in the art.

For example, biochemical pathways competing with the ω-3 and/or ω-6 fatty acid biosynthetic pathways for energy or carbon, or native PUFA biosynthetic pathway enzymes that interfere with production of a particular PUFA end-product,

may be eliminated by gene disruption or down-regulated by other means (e.g., antisense mRNA).

Detailed discussion of manipulations within the PUFA biosynthetic pathway as a means to increase ARA, EPA or DHA (and associated techniques thereof) are presented in U.S. Patent Publication No. 2006-0094092-A1, U.S. Patent Publication No. 2006-0115881-A1 and U.S. Patent Publication No. 2006-0110806-A1, respectively, as are desirable manipulations in the TAG biosynthetic pathway and the TAG degradation pathway (and associated techniques thereof).

Within the context of the present invention, it may be useful to modulate the expression of the fatty acid biosynthetic pathway by any one of the strategies described above. For example, the present invention provides methods whereby genes encoding key enzymes in the Δ9 elongase/Δ8 desaturase biosynthetic pathway are introduced into oleaginous yeasts for the production of ω-3 and/or ω-6 fatty acids. It will be particularly useful to express the present the Δ9 elongase genes in oleaginous yeasts that do not naturally possess ω-3 and/or ω-6 fatty acid biosynthetic pathways and coordinate the expression of these genes, to maximize production of preferred PUFA products using various means for metabolic engineering of the host organism.

Microbial Fermentation Processes For PUFA Production

The transformed microbial host cell is grown under conditions that optimize expression of chimeric desaturase and elongase genes and produce the greatest and most economical yield of desired PUFAs. In general, media conditions that may be optimized include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the amount of different mineral ions, the oxygen level, growth temperature, pH, length of the biomass production phase, length of the oil accumulation phase and the time and method of cell harvest. Microorganisms of interest, such as oleaginous yeast (e.g., *Yarrowia lipolytica*) are generally grown in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)) or a defined minimal media that lacks a component necessary for growth and thereby forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI)).

Fermentation media in the present invention must contain a suitable carbon source. Suitable carbon sources are taught in U.S. Patent 7,238,482. Although it is

contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing sources, preferred carbon sources are sugars (e.g., glucose), glycerol, and/or fatty acids.

Nitrogen may be supplied from an inorganic (e.g., $(NH_4)_2SO_4$) or organic (e.g., urea or glutamate) source. In addition to appropriate carbon and nitrogen sources, the fermentation media must also contain suitable minerals, salts, cofactors, buffers, vitamins and other components known to those skilled in the art suitable for the growth of the oleaginous host and promotion of the enzymatic pathways necessary for PUFA production. Particular attention is given to several metal ions (e.g., Fe^{+2}, Cu^{+2}, Mn^{+2}, Co^{+2}, Zn^{+2}, Mg^{+2}) that promote synthesis of lipids and PUFAs (Nakahara, T. et al., *Ind. Appl. Single Cell Oils,* D. J. Kyle and R. Colin, eds. pp 61-97 (1992)).

Preferred growth media in the present invention are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the transformant host cells will be known by one skilled in the art of microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to pH 8.0, wherein pH 5.5 to pH 7.5 is preferred as the range for the initial growth conditions. The fermentation may be conducted under aerobic or anaerobic conditions, wherein microaerobic conditions are preferred.

Typically, accumulation of high levels of PUFAs in oleaginous yeast cells requires a two-stage process, since the metabolic state must be "balanced" between growth and synthesis/storage of fats. Thus, most preferably, a two-stage fermentation process is necessary for the production of PUFAs in oleaginous yeast (e.g., *Yarrowia lipolytica*). This approach is described in U.S. Patent 7,238,482, as are various suitable fermentation process designs (i.e., batch, fed-batch and continuous) and considerations during growth.

Purification And Processing Of PUFA Oils

PUFAs may be found in the host microorganisms as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cells through a variety of means well-known in the art. One review of extraction techniques, quality analysis and acceptability

standards for yeast lipids is that of Z. Jacobs (*Critical Reviews in Biotechnology,* 12(5/6):463-491 (1992)). A brief review of downstream processing is also available by A. Singh and O. Ward (*Adv. Appl. Microbiol.*, 45:271-312 (1997)).

In general, means for the purification of PUFAs may include extraction (e.g., U.S. Patent 6,797,303 and U.S. Patent 5,648,564) with organic solvents, sonication, supercritical fluid extraction (e.g., using carbon dioxide), saponification and physical means such as presses, or combinations thereof. One is referred to the teachings of U.S. Patent 7,238,482 for additional details.

PUFA-Containing Oils For Use In Foodstuffs, Health Food Products, Pharmaceuticals And Animal Feeds

The market place currently supports a large variety of food and feed products, incorporating ω-3 and/or ω-6 fatty acids (particularly e.g., ALA, GLA, ARA, EPA, DPA and DHA). It is contemplated that the microbial biomass comprising long-chain PUFAs, partially purified microbial biomass comprising PUFAs, purified microbial oil comprising PUFAs, and/or purified PUFAs will function in food and feed products to impart the health benefits of current formulations. More specifically, oils of the invention containing ω-3 and/or ω-6 fatty acids will be suitable for use in a variety of food and feed products including, but not limited to: food analogs, meat products, cereal products, baked foods, snack foods and dairy products (see Patent Publication No. US-2006-0094092 for details).

Additionally, the present compositions may be used in formulations to impart health benefit in medical foods including medical nutritionals, dietary supplements, infant formula as well as pharmaceutical products. One of skill in the art of food processing and food formulation will understand how the amount and composition of the present oils may be added to the food or feed product. Such an amount will be referred to herein as an "effective" amount and will depend on the food or feed product, the diet that the product is intended to supplement or the medical condition that the medical food or medical nutritional is intended to correct or treat.

EXAMPLES

The present invention is further defined in the following Examples, in which parts and percentages are by weight and degrees are Celsius, unless otherwise stated. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above

discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions. Thus, various modifications of the invention in addition to those shown and described herein will be apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

GENERAL METHODS

Standard recombinant DNA and molecular cloning techniques used in the Examples are well known in the art and are described by: 1.) Sambrook, J., Fritsch, E. F. and Maniatis, T. *Molecular Cloning: A Laboratory Manual*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (Maniatis); 2.) T. J. Silhavy, M. L. Bennan, and L. W. Enquist, Experiments with Gene Fusions; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and 3.) Ausubel, F. M. et al., Current Protocols in Molecular Biology, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, Eds), American Society for Microbiology: Washington, D.C. (1994)); or by Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of microbial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified. *E. coli* strains were typically grown at 37 °C on Luria Bertani (LB) plates.

General molecular cloning was performed according to standard methods (Sambrook et al., *supra*). DNA sequence was generated on an ABI Automatic sequencer using dye terminator technology (U.S. Patent No. 5,366,860; EP 272,007) using a combination of vector and insert-specific primers. Sequence editing was performed in Sequencher (Gene Codes Corporation, Ann Arbor, MI). All

sequences represent coverage at least two times in both directions. Comparisons of genetic sequences were accomplished using DNASTAR software (DNASTAR Inc., Madison, WI).

The meaning of abbreviations is as follows: "sec" means second(s), "min" means minute(s), "h" or "hr" means hour(s), "d" means day(s), "µL" means microliter(s), "mL" means milliliter(s), "L" means liter(s), "µM" means micromolar, "mM" means millimolar, "M" means molar, "mmol" means millimole(s), "µmole" mean micromole(s), "g" means gram(s), "µg" means microgram(s), "ng" means nanogram(s), "U" means unit(s), "bp" means base pair(s) and "kB" means kilobase(s).

Nomenclature For Expression Cassettes:

The structure of an expression cassette will be represented by a simple notation system of "X::Y::Z", wherein X describes the promoter fragment, Y describes the gene fragment, and Z describes the terminator fragment, which are all operably linked to one another.

Transformation And Cultivation Of *Yarrowia lipolytica*:

Yarrowia lipolytica strains with ATCC Accession Nos. #20362, #76982 and #90812 were purchased from the American Type Culture Collection (Rockville, MD). *Yarrowia lipolytica* strains were typically grown at 28-30 °C in several media, according to the recipes shown below. Agar plates were prepared as required by addition of 20 g/L agar to each liquid media, according to standard methodology.

YPD agar medium (per liter): 10 g of yeast extract [Difco], 20 g of Bacto peptone [Difco]; and 20 g of glucose.

Basic Minimal Media (MM) (per liter): 20 g glucose; 1.7 g yeast nitrogen base without amino acids; 1.0 g proline; and pH 6.1 (not adjusted).

Minimal Media + 5-Fluoroorotic Acid (MM + 5-FOA) (per liter): 20 g glucose, 6.7 g Yeast Nitrogen base, 75 mg uracil, 75 mg uridine and appropriate amount of FOA (Zymo Research Corp., Orange, CA), based on FOA activity testing against a range of concentrations from 100 mg/L to 1000 mg/L (since variation occurs within each batch received from the supplier).

Transformation of *Yarrowia lipolytica* was performed according to the method of Chen, D. C. et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)), unless otherwise noted. Briefly, *Yarrowia* was streaked onto a YPD plate and grown at 30 °C for approximately 18 h. Several large loopfuls of cells were scraped from the plate and resuspended in 1 mL of transformation buffer, comprising: 2.25 mL of 50% PEG, average MW 3350; 0.125 mL of 2 M lithium acetate, pH 6.0; 0.125 mL of 2 M DTT; and (optionally) 50 μg sheared salmon sperm DNA. Then, approximately 500 ng of linear DNA (preferably comprising at least one chimeric gene) (or 100 ng circular plasmid) was incubated in 100 μL of resuspended cells, and maintained at 39 °C for 1 h with vortex mixing at 15 min intervals. The cells were plated onto selection media plates and maintained at 30 °C for 2 to 3 days.

Fatty Acid Analysis Of *Yarrowia lipolytica*:

For fatty acid analysis, cells were collected by centrifugation and lipids were extracted as described in Bligh, E. G. & Dyer, W. J. (*Can. J. Biochem. Physiol.*, 37:911-917 (1959)). Fatty acid methyl esters were prepared by transesterification of the lipid extract with sodium methoxide (Roughan, G. and Nishida I., *Arch Biochem Biophys.,* 276(1):38-46 (1990)) and subsequently analyzed with a Hewlett-Packard 6890 GC fitted with a 30 m X 0.25 mm (i.d.) HP-INNOWAX (Hewlett-Packard) column. The oven temperature was from 170 °C (25 min hold) to 185 °C at 3.5 °C/min.

For direct base transesterification, *Yarrowia* culture (3 mL) was harvested, washed once in distilled water, and dried under vacuum in a Speed-Vac for 5-10 min. Sodium methoxide (100 μL of 1%) was added to the sample, and then the sample was vortexed and rocked for 20 min. After adding 3 drops of 1 M NaCl and 400 μL hexane, the sample was vortexed and spun. The upper layer was removed and analyzed by GC as described above.

EXAMPLE 1

Synthesis Of A cDNA Library From *Euglena anabaena* UTEX 373

The present Example describes the synthesis of a cDNA library from *Euglena anabaena* UTEX 373. This work included the preparation of RNA, synthesis of cDNA, and generation of a cDNA library.

Growth Of *Euglena anabaena* UTEX 373 And Preparation Of RNA

Euglena anabaena UTEX 373 was obtained from Dr. Richard Triemer's lab at Michigan State University (East Lansing, MI). Approximately 2 mL of culture was removed for lipid analysis and centrifuged at 1,800 x g for 5 min. The pellet was washed once with water and re-centrifuged. The resulting pellet was dried for 5 min under vacuum, resuspended in 100 μL of trimethylsulfonium hydroxide (TMSH) and incubated at room temperature for 15 min with shaking. After incubation, 0.5 mL of hexane was added and the vials were further incubated for 15 min at room temperature with shaking. Fatty acid methyl esters (5 μL injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Supelco Inc., Catalog No. 24152). The oven temperature was programmed to hold at 170 °C for 1.0 min, increase to 240 °C at 5 °C /min and then hold for an additional 1.0 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc. Catalog No. U-99-A) and the resulting chromatogram is shown in FIG. 2. The presence of EDA, ETrA, EPA and DHA in the fatty acid profile, with the absence of GLA and STA, suggested that *Euglena anabaena* uses the Δ9 elongase/Δ8 desaturase pathway for long-chain (LC) PUFA biosynthesis and would be a good source for LC-PUFA biosynthetic genes such as, but not limited to, Δ9 elongases.

The remaining 5 mL of an actively growing culture was transferred into 25 mL of AF-6 Medium (Watanabe & Hiroki, NIES-Collection List of Strains, 5th ed., National Institute for Environmental Studies, Tsukuba, 127 pp (2004)) in a 125 mL glass flask. *Euglena anabaena* cultures were grown at 22 °C with a 16 h light, 8 h dark cycle for 2 weeks with very gentle agitation.

After 2 weeks, the culture (25 mL) was transferred to 100 mL of AF-6 medium in a 500 mL glass bottle and the culture was grown for 1 month as described above. After this time, two 50 mL aliquots were transferred into two separate 500 mL glass bottles containing 250 mL of AF-6 medium and the cultures were grown for two months as described above (giving a total of ~600 mL of culture). Next, the cultures were pelleted by centrifugation at 1,800 x g for 10 min, washed once with water and re-centrifuged. Total RNA was extracted from one of the resulting pellets using the

RNA STAT-60™ reagent (TEL-TEST, Inc., Friendswood, TX) and following the manufacturer's protocol provided (use 5 mL of reagent, dissolved RNA in 0.5 mL of water). In this way, 340 μg of total RNA (680 μg/mL) was obtained from the pellet. The remaining pellet was frozen in liquid nitrogen and stored at -80 °C. The mRNA was isolated from all 340 μg of total RNA using the mRNA Purification Kit (Amersham Biosciences, Piscataway, NJ) following the manufacturer's protocol provided. In this way, 9.0 μg of mRNA was obtained.

Preparation Of *Euglena anabaena* cDNA And Generation Of cDNA Library eug1c

A cDNA library was generated using the Cloneminer™ cDNA Library Construction Kit (Catalog No.18249-029, Invitrogen Corporation, Carlsbad, CA) and following the manufacturer's protocol provided (Version B, 25-0608). Using the non-radiolabeling method, cDNA was synthesized from 5.12 μg of mRNA (described above) using the Biotin-*att*B2-Oligo(dT) primer. After synthesis of the first and second strand, the *att*B1 adapter was added, ligated and the cDNA was size fractionated using column chromatography. DNA from fractions were concentrated, recombined into pDONR™222 and transformed into *E. coli* ElectroMAX™ DH10B™ T1 Phage-Resistant cells (Invitrogen Corporation). The *Euglena anabaena* library was named eug1c.

The cDNA library eug1c was plated onto LB +Kanamycin plates (approx. 100,000 colonies), the colonies were scraped off and DNA was isolated using the QIAprep® Spin Miniprep Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. In this way, a plasmid DNA sub-library from eug1c was obtained.

EXAMPLE 2

Isolation Of Full-Length Δ9 Elongases From *Euglena anabaena* UTEX 373

The present Example describes the identification of cDNAs (SEQ ID NOs:1 and 2) encoding Δ9 elongases from *Euglena anabaena* UTEX 373. This work included the generation of a probe derived from the *Euglena gracilis* Δ9 elongase (EgD9e; SEQ ID NO:3) and the hybridization of the probe to the cDNA library eug1c in order to identify Δ9 elongase homologs from *Euglena anabaena* UTEX 373.

Euglena gracilis Δ9 Elongase (EgD9e)

A clone from the *Euglena* cDNA library (eeg1c), called eeg1c.pk001.n5f, containing the *Euglena gracilis* Δ9 elongase (EgD9e; SEQ ID NO:3; which is described in U.S. Application No. 11/601,563 was used as template to amplifiy EgD9e with oligonucleotide primers oEugEL1-1 (SEQ ID NO:5) and oEugEL1-2 (SEQ ID NO:6) using the VentR® DNA Polymerase (Catalog No. M0254S, New England Biolabs Inc., Beverly, MA) following the manufacturer's protocol. The resulting DNA fragment was cloned into the pCR-Blunt® cloning vector using the Zero Blunt® PCR Cloning Kit (Invitrogen Corporation), following the manufacturer's protocol, to produce pKR906 (SEQ ID NO:15).

Colony Lifts

Approximately 17,000 clones of *Euglena anabaena* cDNA library eug1c were plated onto three large square (24 cm x 24 cm) petri plates (Corning, Corning, NY) each containing LB + 50 µg/mL kanamycin agar media. Cells were grown overnight at 37 °C and plates were then cooled to room temperature.

Biodyne B 0.45 µm membrane (Catalog No. 60207, Pall Corporation, Pensacola, FL) was trimmed to approximately 22 cm x 22 cm and the membrane was carefully layed on top of the agar to avoid air bubbles. After incubation for 2 min at room temperature, the membrane was marked for orientation, lifted off with tweezers and placed colony-side up on filter paper soaked with 0.5 M sodium hydroxide and 1.5 M sodium chloride. After denaturation for 4 min, the sodium hydroxide was neutralized by placing the membrane on filter paper soaked with 0.5 M Tris-HCL (pH 7.5) and 1.5 M sodium chloride for 4 min. This step was repeated and the membrane was rinsed briefly in 2X SSC buffer (20X SSC is 3 M sodium chloride, 0.3 M sodium citrate; pH 7.0) and air dried on filter paper.

Hybridization

Membranes were pre-hybridized at 65 °C in 200 mL hybridization solution for 2 hr. Hybridization solution contained 6X SSPE (20X SSPE is 3 M sodium chloride, 0.2 M sodium phosphate, 20 mM EDTA; pH 7.4), 5X Denhardt's reagent (100X Denhardt's reagent is 2%(w/v) Ficoll, 2% (w/v) polyvinylpyrrolidone, 2% (w/v) acetylated bovine serum albumin), 0.5% sodium dodecyl sulfate (SDS), 100 µg/mL sheared salmon sperm DNA and 5% dextran sulfate.

A DNA probe was made using an agarose gel purified *Nco*I/*Not*I DNA fragment, containing the *Euglena gracilis* Δ9 elongase gene, from pKR906 (SEQ ID NO:15) labeled with P^{32} dCTP using the RadPrime DNA Labeling System (Catalog No. 18428-011, Invitrogen, Carlsbad, CA) following the manufacturer's instructions. Unincorporated P^{32} dCTP was separated using a NICK column (Catalog No. 17-0855-02, Amersham Biosciences, Piscataway, NJ) following the manufacturer's instructions. The probe was denatured for 5 min at 100 °C, placed on ice for 3 min and half was added to the hybridization solution.

The membrane was hybridized with the probe overnight at 65 °C with gentle shaking and then washed the following day twice with 2X SSC containing 0.5% SDS (5 min each) and twice with 0.2X SSC containing 0.1% SDS (15 min each). After washing, hyperfilm (Catalog No. RPN30K, Amersham Biosciences, Piscataway, NJ) was exposed to the membrane overnight at -80 °C.

Based on alignment of plates with the exposed hyperfilm, positive colonies were picked using the blunt end of a Pasteur pipette into 1 mL of water and vortexed. Several dilutions were made and plated onto small round Petri dishes (82 mm) containing LB media plus 50 µg/mL kanamycin to obtain around 100 well isolated colonies on a single plate. Lifts were done as described above except NytranN membrane circles (Catalog No. 10416116, Schleicher & Schuell, Keene, NH) were used and hybridization was carried out in 100 mL using the remaining radiolabeled probe. In this way, positive clones were confirmed.

Individual positive clones were grown at 37 °C in LB + 50 µg/mL kanamycin liquid media and plasmid was purified using the QIAprep® Spin Miniprep Kit (Qiagen Inc.) following the manufacturer's protocol.

DNA inserts were end-sequenced in 384-well plates, using vector-primed M13F universal primer (SEQ ID NO:7), M13rev-28 primer (SEQ ID NO:8) and the poly(A) tail-primed WobbleT oligonucleotides, with the ABI BigDye version 3 Prism sequencing kit. For the sequencing reaction, 100-200 ng of template and 6.4 pmol of primer were used, and the following reaction conditions were repeated 25 times: 96 °C for 10 sec, 50 °C for 5 sec and 60 °C for 4 min. After ethanol-based cleanup, cycle sequencing reaction products were resolved and detected on Perkin-Elmer ABI 3700 automated sequencers. The WobbleT primer is an equimolar mix of

21mer poly(T)A, poly(T)C, and poly(T)G, used to sequence the 3' end of cDNA clones.

Sequences were aligned and compared using Sequencher™ (Version 4.2, Gene Codes Corporation, Ann Arbor, MI) and in this way, the clones could be categorized into one of two distinct groups based on insert sequence (designated as EaD9Elo1 and EaD9Elo2). Representative clones containing the cDNA for each class of sequence were chosen for further study and sequences for each representative plasmid (i.e., pLF121-1 and pLF121-2) are shown in SEQ ID NO:9 and SEQ ID NO:10, respectively. The sequence shown by a string of NNNN's represents a region of the polyA tail which was not sequenced. The coding sequences for EaD9Elo1 and EaD9Elo2 are shown in SEQ ID NO:11 and SEQ ID NO:12, respectively. The corresponding amino acid sequences for EaD9Elo1 and EaD9Elo2 are shown in SEQ ID NO:13 and SEQ ID NO:14, respectively.

EXAMPLE 3

Primary Sequence Analysis Of The Δ9 Elongase Sequences Of *Euglena anabaena* UTEX 373 (EaD9Elo1 And EaD9Elo2) And Comparison To Other Published Δ9 Elongase Sequences

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were compared using the Clustal V method (Higgins, D.G. and Sharp, P.M., *Comput. Appl. Biosci.,* 5:151-153 (1989); Higgins et al., *Comput. Appl. Biosci.,* 8:189-191 (1992)) using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5 and GAP LENGTH PENALTY=10).

Compared to EaD9Elo1 (SEQ ID NO:13), EaD9Elo2 (SEQ ID NO:14) has 1 amino acid substitution (i.e., R254Q; based on numbering for EaD9Elo1). The nucleotide sequences of EaD9Elo1 (SEQ ID NO:11) and EaD9Elo2 (SEQ ID NO:12) differ by six base pairs over the full 774 bp lengths.

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were evaluated by BLASTP (Basic Local Alignment Search Tool; Altschul et al., *J. Mol. Biol.,* 215:403-410 (1993)) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure

Brookhaven Protein Data Bank, the last major release of the SWISS-PROT protein sequence database, EMBL and DDBJ databases) using default parameters with the filter turned off. For convenience, the P-value (probability) of observing a match of a cDNA sequence to a sequence contained in the searched databases merely by chance as calculated by BLAST are reported herein as "pLog" values, which represent the negative of the logarithm of the reported P-value. Accordingly, the greater the pLog value, the greater the likelihood that the cDNA sequence and the BLAST "hit" represent homologous proteins.

Both sequences yielded a pLog value of 38.70 (P value of 2e-39) versus the *Isochrysis galbana* long chain polyunsaturated fatty acid elongation enzyme (IgD9e; SEQ ID NO:16) (NCBI Accession No. AAL37626(GI 17226123), locus AAL37626, CDS AF390174 ; Qi et al., *FEBS Lett.,* 510:159-165 (2002)) when compared to the "nr" database. BLAST scores and probabilities indicate that the instant nucleic acid fragments encode entire *Euglena anabaena* Δ9 fatty acid elongases.

The amino acid sequences for EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) were compared to IgD9e (SEQ ID NO:16) and the *Euglena gracilis* Δ9 elongase amino acid sequence (EgD9e; SEQ ID NO:4; PCT Publication No. WO 2007/061845) using BlastP, Clustal V and the Jotun Hein methods of sequence comparison. The % identity against IgD9e and EgD9e using each method is shown in Table 4 and Table 5, respectively.

Sequence percent identity calculations were performed by the BlastP and Clustal V methods, as described above. Sequence percent identity calculations performed by the Jotun Hein method (Hein, J. J., *Meth. Enz,* 183:626-645 (1990)) were done using the MegAlign™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI) with the default parameters for pairwise alignment (KTUPLE=2).

Table 4

Sequence Comparison Of EaD9Elo1 (SEQ ID NO:13) And Ead9elo2 (SEQ ID NO:14) To IgD9e (SEQ ID NO:16)

Desaturase	% Identity to IgD9e by BLASTP	% Identity to IgD9e by the Jotun Hein Method	% Identity to IgD9e by the Clustal V Method
EaD9Elo1	37%	40.4%	32.9%
EaD9Elo2	37%	41.2%	32.9%

Table 5

Sequence Comparison Of EaD9Elo1 (SEQ ID NO:13) And EaD9Elo2 (SEQ ID NO:14) To EgD9e (SEQ ID NO:4)

Desaturase	% Identity to EgD9e by BLASTP	% Identity to EgD9e by the Jotun Hein Method	% Identity to EgD9e by the Clustal V Method
EaD9Elo1	77%	77.2%	77.1%
EaD9Elo2	77%	77.2%	77.1%

EXAMPLE 4

Functional Analysis Of The *Euglena anabaena* UTEX 373 Δ9 Elongases In *Yarrowia lipolytica*

The present Example describes functional analysis of EaD9Elo1 (SEQ ID NO:13) and EaD9Elo2 (SEQ ID NO:14) in *Yarrowia lipolytica.* This work included the following steps: (1) Construction of Gateway®-compatible *Yarrowia* expression vector pY159; (2) transfer of EaD9Elo1 and EaD9Elo2 into pY159 to produce pY173 and pY174; and, (3) comparison of lipid profiles within transformant organisms comprising pY173 and pY174.

Construction Of Gateway®-Compatible *Yarrowia* Expression Vector pY159

Plasmid pY5-30 (which was previously described in U.S. Patent 7,259,255), is a shuttle plasmid that can replicate both in *E. coli* and *Yarrowia lipolytica*. Plasmid pY5-30 contains the following: a *Yarrowia* autonomous replication sequence (ARS18); a ColE1 plasmid origin of replication; an ampicillin-resistance gene (Amp^R), for selection in *E. coli*; a *Yarrowia* LEU2 gene, for selection in

Yarrowia; and a chimeric TEF::GUS::XPR gene. Plasmid pDMW263 (SEQ ID NO:17) was created from pY5-30, by replacing the TEF promoter with the *Yarrowia lipolytica* FBAINm promoter (U.S. Patent 7,202,356) using techniques well known to one skilled in the art. Briefly, this promoter refers to a modified promoter which is located in the 5' upstream untranslated region in front of the 'ATG' translation initiation codon of the fructose-bisphosphate aldolase enzyme (E.C. 4.1.2.13) encoded by the *fba1* gene and that is necessary for expression, plus a portion of 5' coding region that has an intron, wherein FBAINm has a 52 bp deletion between the ATG translation initiation codon and the intron of the FBAIN promoter (thereby including only 22 amino acids of the N-terminus) and a new translation consensus motif after the intron. Table 6 summarizes the components of pDMW263 (SEQ ID NO:17).

Table 6

Components Of Plasmid pDMW263 (SEQ ID NO:17)

RE Sites and Nucleotides Within SEQ ID NO:17	Description of Fragment and Chimeric Gene Components
4992-4296	ARS18 sequence (GenBank Accession No. A17608)
SalI/SacII (8505-2014)	FBAINm::GUS::XPR, comprising: • FBAINm: *Yarrowia lipolytica* FBAINm promoter (U.S. Patent 7,202,356); • GUS: *E. coli* gene encoding β-glucuronidase (Jefferson, R.A. *Nature,* 14:342:837-838 (1989); • XPR: ~100 bp of the 3' region of the *Yarrowia Xpr* gene (GenBank Accession No. M17741)
6303-8505	*Yarrowia Leu2* gene (GenBank Accession No. AF260230)

The *Nco*I/*Sal*I DNA fragment from pDMW263 (SEQ ID NO:17), containing the *Yarrowia lipolytica* FBAINm promoter, was cloned into the *Nco*I/*Sal*I DNA fragment of pDMW237 (SEQ ID NO:18), previously described in PCT Publication No. WO 2006/012325 (the contents of which are hereby incorporated by reference), containing a synthetic Δ9 elongase gene derived from *Isochrysis galbana* and codon-optimized for expression in *Yarrowia lipolytica* (IgD9eS), to produce pY115 (SEQ ID NO:19; FIG. 3A). In FIG. 3A, the modified FBAINm promoter is labeled as

FBA1 + Intron, while it is labeled as YAR FBA1 PRO + Intron in FIGs. 3B, 3C and 3D.

The FBAINm promoter was amplified from plasmid pY115 (SEQ ID NO:19), using PCR with oligonucleotide primers oYFBA1 (SEQ ID NO:20) and oYFBA1-6 (SEQ ID NO:21). Primer oYFBA1 (SEQ ID NO:20) was designed to introduce a *Bgl*II site at the 5' end of the promoter and primer oYFBA1-6 (SEQ ID NO:21) was designed to introduce a *Not*I site at the 3' end of the promoter while removing the *Nco*I site and thus, the ATG start codon. The resulting PCR fragment was digested with *Bgl*II and *Not*I and cloned into the *Bgl*II/*Not*I fragment of pY115, containing the vector backbone, to form pY158 (SEQ ID NO:22).

Plasmid pY158 (SEQ ID NO:22) was digested with *Not*I and the resulting DNA ends were filled. After filling to form blunt ends, the DNA fragments were treated with calf intestinal alkaline phosphatase and separated using agarose gel electrophoresis. The 6992 bp fragment containing the *Yarrowia lipolytica* FBAINm promoter was excised from the agarose gel and purified using the QIAquick® Gel Extraction Kit (Qiagen Inc., Valencia, CA) following the manufacturer's protocol. The purified 6992 bp fragment was ligated with cassette rfA using the Gateway Vector Conversion System (Catalog No. 11823-029, Invitrogen Corporation) following the manufacturer's protocol to form *Yarrowia lipolytica* Gateway® destination vector pY159 (SEQ ID NO:23; FIG. 3B).

Construction Of Yarrowia Expression Vectors pY173 And pY174

Using the Gateway® LR Clonase™ II enzyme mix (Catalog No. 11791-020, Invitrogen Corporation) and following the manufacturer's protocol, the cDNA inserts from pLF121-1 (SEQ ID NO:9; Example 2) and pLF121-2 (SEQ ID NO:10; Example 2) were transferred to pY159 (SEQ ID NO:23) to form pY173 (SEQ ID NO:24; FIG. 3C) and pY174 (SEQ ID NO:25; FIG. 3D), respectively.

Functional Analysis Of EaD9Elo1 And EaD9Elo2 In *Yarrowia lipolytica* Strain Y2224

Strain Y2224 was isolated in the following manner: *Yarrowia lipolytica* ATCC #20362 cells from a YPD agar plate (1% yeast extract, 2% bactopeptone, 2% glucose, 2% agar) were streaked onto a MM plate (75 mg/L each of uracil and uridine, 6.7 g/L YNB with ammonia sulfate, without amino acid, and 20 g/L glucose) containing 250 mg/L 5-FOA (Zymo Research). Plates were incubated at 28 °C and four of the resulting colonies were patched separately onto MM plates containing

200 mg/mL 5-FOA and MM plates lacking uracil and uridine to confirm uracil *Ura3* auxotrophy.

Strain Y2224 was transformed with pY173 (SEQ ID NO:24; FIG. 3C) and pY174 (SEQ ID NO:25; FIG. 3D) as described in the General Methods.

Single colonies of transformant *Yarrowia lipolytica* containing pY173 and pY174 were grown in 3 mL MM lacking uracil at 30 °C for 16 h after which cells were centrifuged at 250 rpm to pellet. Cells were washed once with water, pelleted by centrifugation and air dried. Pellets were transesterified (Roughan, G. and Nishida, I., *Arch. Biochem. Biophys.,* 276(1):38-46 (1990)) with 500 µL of 1% sodium methoxide for 30 min at 50 °C after which 500 µL of 1 M sodium chloride and 100 µL of heptane were added. After thorough mixing and centrifugation, fatty acid methyl esters (FAMEs) were analyzed by GC. FAMEs (5 µL injected from hexane layer) were separated and quantified using a Hewlett-Packard 6890 Gas Chromatograph fitted with an Omegawax 320 fused silica capillary column (Catalog No. 24152, Supelco Inc.). The oven temperature was programmed to hold at 220 °C for 2.6 min, increase to 240 °C at 20 °C/min and then hold for an additional 2.4 min. Carrier gas was supplied by a Whatman hydrogen generator. Retention times were compared to those for methyl esters of standards commercially available (Nu-Chek Prep, Inc.).

The fatty acid profiles for *Yarrowia lipolytica* expressing pY173 and pY174 are shown in Table 7. Fatty acids are identified as 16:0, 16:1, 18:0, 18:1 (oleic acid), LA, 20:0, 20:1(11), EDA, 22:0, 24:0 and 24:1. Percent Δ9 elongation (Δ9 %Elong) was calculated by dividing the weight % (wt %) for EDA by the sum of the wt % for EDA and LA and multiplying by 100 to express as a %. Average is indicated by Ave.

Table 7

Fatty Acid Composition (Wt %) For *Yarrowia lipolytica* Expressing pY173 (EaD9Elo1) And pY174 (EaD9Elo2)

Event	16:0	16:1	18:0	18:1	LA	20:0	20:1 (11)	EDA	22:0	24:0	24:1	Δ9 %Elong	Ave. Δ9 %Elong
Y173-1	16.7	14.5	4.1	46.5	12.5	0.2	0.2	3.6	0.2	1.4	0.1	22.2	
Y173-2	16.6	14.2	4.1	46.8	12.4	0.2	0.2	3.7	0.2	1.5	0.1	22.7	22.7
Y173-3	16.5	14.0	4.2	47.1	12.3	0.2	0.2	3.7	0.2	1.5	0.2	23.2	
Y174-1	16.9	14.3	4.2	46.8	12.5	0.2	0.2	3.2	0.2	1.4	0.1	20.5	
Y174-2	17.0	14.1	4.3	47.4	11.8	0.2	0.2	3.3	0.2	1.4	0.1	21.6	21.1
Y174-3	17.0	14.2	4.3	47.2	11.9	0.2	0.2	3.2	0.2	1.4	0.2	21.2	

EXAMPLE 5

Synthesis Of A Codon-Optimized Δ9 Elongase Gene For *Yarrowia lipolytica* (EaD9ES)

The codon usage of the Δ9 elongase gene (EaD9Elo1) of *Euglena anabaena* was optimized for expression in *Yarrowia lipolytica*, in a manner similar to that described in PCT Publication No. WO 2004/101753 and U.S. Patent 7,125,672. Specifically, a codon-optimized Δ9 elongase gene (designated "EaD9ES"; SEQ ID NO:26) was designed based on the coding sequence of EaD9Elo1 (SEQ ID NO:11), according to the *Yarrowia* codon usage pattern (PCT Publication No. WO 2004/101753), the consensus sequence around the 'ATG' translation initiation codon, and the general rules of RNA stability (Guhaniyogi, G. and J. Brewer, *Gene*, 265(1-2):11-23 (2001)). In addition to modification of the translation initiation site, 106 bp of the 774 bp coding region were modified (13.7%) and 98 codons were optimized (38.0%). The GC content (52.1%) was about the same between the wild type gene (i.e., EaD9Elo1) and the synthetic gene (i.e., EaD9ES). A *Nco*I site and *Not*I sites were incorporated around the translation initiation codon and after the stop codon of EaD9ES (SEQ ID NO:26), respectively. FIGs. 4A and 4B show a comparison of the nucleotide sequences of EaD9Elo1 (SEQ ID NO:11) and EaD9ES (SEQ ID NO:26). The protein sequence encoded by the codon-optimized gene (i.e., SEQ ID NO:27) is identical to that of the wildtype protein sequence (i.e., SEQ ID NO:13). The designed EaD9ES gene was synthesized by GenScript Corporation (Piscataway, NJ) and cloned into pUC57 (GenBank Accession No. Y14837) to generate pEaD9ES (SEQ ID NO:28; FIG. 5A).

EXAMPLE 6

Construction And Functional Analysis Of *Yarrowia lipolytica* Expression Vector pZUFmEaD9ES, Comprising A Synthetic Δ9 Elongase Gene (Derived From *Euglena anabaena*), Codon-Optimized For Expression In *Yarrowia lipolytica* (EaD9ES)

The present Example describes the functional expression of *Yarrowia lipolytica* vector pZUFmEaD9ES, comprising a chimeric FBAINm::EaD9ES::Pex20 gene, wherein EaD9ES is a synthetic Δ9 elongase derived from *Euglena anabaena* and codon-optimized for expression in *Yarrowia*. The plasmid pZUFmEaD9ES (FIG. 5B) contained the following components:

Table 8

Components Of Plasmid pZUFmEaD9ES (SEQ ID NO:29)

RE Sites And Nucleotides Within SEQ ID NO:29	Description Of Fragment And Chimeric Gene Components
Swa I/BsiW I (6067-318)	FBAINm::EaD9ES::Pex20, comprising: • FBAINm: *Yarrowia lipolytica* FBAIN promoter (U.S. Patent 7,202,356) • EaD9ES: codon-optimized Δ9 elongase (SEQ ID NO:26), derived from *Euglena anabaena* • Pex20: Pex20 terminator sequence of *Yarrowia* Pex20 gene (GenBank Accession No. AF054613)
1354-474	ColE1 plasmid origin of replication
2284-1424	Ampicillin-resistance gene (Amp^R) for selection in *E. coli*
3183-4487	*Yarrowia* autonomous replication sequence (ARS18; GenBank Accession No. A17608)
6031-4530	*Yarrowia Ura 3* gene (GenBank Accession No. AJ306421)

Functional Analysis Of *Yarrowia lipolytica* Transformants Comprising pZUFmEaD9ES

Plasmid pZUFmEaD9ES was transformed into strain Y2224 (the FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362), as described in the General Methods. The transformants were selected on MM plates. After 2 days growth at 30 °C, transformants were picked and re-streaked onto fresh MM plates. Once grown, these strains were individually inoculated into 3 mL liquid MM at 30 °C and shaken at 250 rpm/min for 2 days. The cells were collected by centrifugation, lipids were extracted, and fatty acid methyl esters were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that there were about 2.2% C20:2 (EDA) and 15.3% C18:2 (LA) of total lipids produced in all 5 transformants, wherein the conversion efficiency of C18:2 to C20:2 in these 5 strains was determined to be about 13%. Thus, this experimental data demonstrated that the synthetic *Euglena anabaena* Δ9 elongase codon-optimized for expression in *Yarrowia lipolytica* (i.e., EaD9ES, as set forth in SEQ ID NOs:26 and 27) actively elongates LA to EDA.

Where the terms "comprise", "comprises", "comprised" or "comprising" are used in this specification, they are to be interpreted as specifying the presence of the stated features, integers, steps or components referred to, but not to preclude the presence or addition of one or more other feature, integer, step, component or group thereof.

Further, any prior art reference or statement provided in the specification is not to be taken as an admission that such art constitutes, or is to be understood as constituting, part of the common general knowledge in Australia.

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THE CLAIMS DEFINING THE INVENTION ARE AS FOLLOWS:

1. A transformed microbial host cell comprising a non-native polynucleotide encoding a polypeptide having delta-9 elongase activity, wherein the polypeptide comprises an amino acid sequence that is at least 90% identical to SEQ ID NO:13 or SEQ ID NO:14 , based on the Clustal V method of alignment.

2. The microbial host cell of Claim 1, wherein the polypeptide comprises an amino acid sequence as set forth in SEQ ID NO:13 or SEQ ID NO:14.

3. The microbial host cell of Claim 2, wherein the non-native polynucleotide comprises SEQ ID NO:11, SEQ ID NO:12, or SEQ ID NO:26.

4. The microbial host cell of any one of Claims 1-3 selected from the group consisting of yeast, algae, bacteria, euglenoids, stramenopiles and fungi.

5. The microbial host cell of Claim 4, wherein the cell is a fungus of the genus *Mortierella*.

6. The microbial host cell of Claim 4, wherein the cell is a stramenopile selected from the group consisting of: *Thraustochytrium* sp. and *Schizochytrium* sp.

7. The microbial host cell of Claim 4, wherein the cell is a yeast.

8. The microbial host cell of Claim 7, wherein the yeast is an oleaginous yeast.

9. The microbial host cell of Claim 8, wherein the oleaginous yeast is selected from the group consisting of: *Yarrowia, Candida, Rhodotorula, Rhodosporidium*, *Cryptococcus, Trichosporon* and *Lipomyces*.

10. The microbial host cell of Claim 9, wherein the oleaginous yeast is a *Yarrowia lipolytica*.

11. A method for the production of eicosadienoic acid comprising:
a) providing a microbial host cell according to any one of claims 1-10, wherein the cell further comprises a source of linoleic acid;
b) growing the microbial host cell of step (a) under conditions wherein the polynucleotide encoding the polypeptide having delta-9 elongase activity is expressed and the linoleic acid is converted to eicosadienoic acid; and
c) optionally recovering the eicosadienoic acid of step (b).

12. A method for the production of eicosatrienoic acid comprising:
a) providing a microbial host cell according to any one of claims 1-10, wherein the cell further comprises a source of alpha-linolenic acid;
b) growing the microbial host cell of step (a) under conditions wherein the polynucleotide encoding the polypeptide having delta-9 elongase activity is expressed and the alpha-linolenic acid is converted to eicosatrienoic acid; and
c) optionally recovering the eicosatrienoic acid of step (b).

13 The method of either of Claims 11 or 12, wherein (i) the microbial host cell is a *Yarrowia* sp., (ii) the polypeptide comprises SEQ ID NO:27, and (iii) the polynucleotide comprises at least 98 codons that are optimized for expression in *Yarrowia*.

14. The method according to either of Claims 11 or 12, wherein the polynucleotide comprises a nucleic acid sequence selected from the group consisting of SEQ ID NO:11, SEQ ID NO:12 and SEQ ID NO:26; and the host cell is a *Yarrowia lipolytica*.

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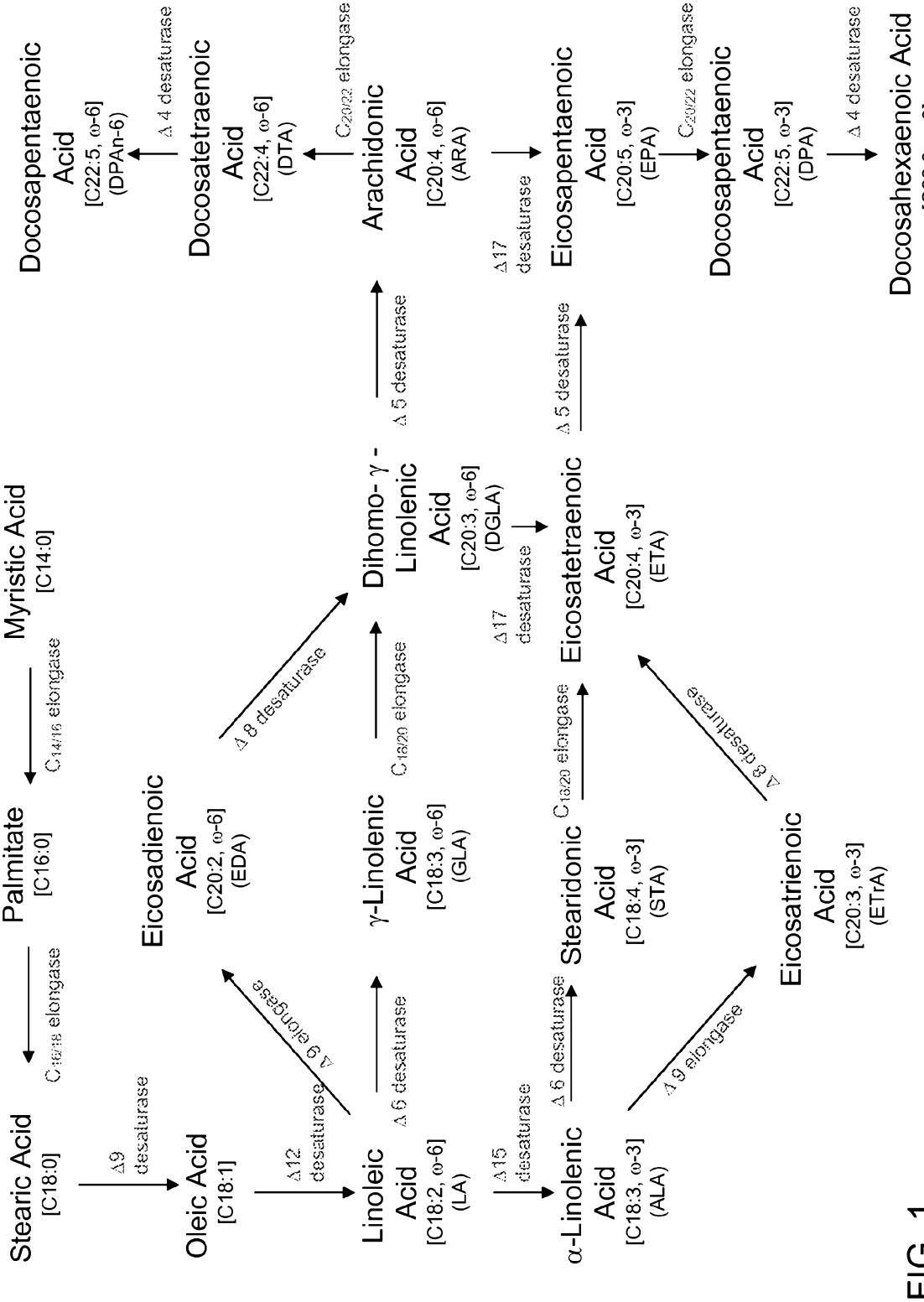
15. An isolated nucleic acid molecule comprising a nucleotide sequence encoding a delta-9 elongase having an amino acid sequence as set forth in SEQ ID NO:27, wherein at least 98 codons of the nucleotide sequence are codon-optimized for expression in a *Yarrowia* sp.

16. The microbial host cell of any one of Claims 1-10, substantially as hereinbefore described.

17. The method of any one of Claims 11-14, substantially as hereinbefore described.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Palmitate
[C16:0]
Myristic Acid
[C14:0]
Oleic Acid
[C18:1]
Eicosadienoic Acid
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(EDA)
Linoleic Acid
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(LA)
γ-Linolenic Acid
[C18:3, ω-6]
(GLA)
Dihomo- γ - Linolenic Acid
[C20:3, ω-6]
(DGLA)
α-Linolenic Acid
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(ALA)
Stearidonic Acid
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(STA)
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(ETrA)
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(ETA)
Arachidonic Acid
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(ARA)
Eicosapentaenoic Acid
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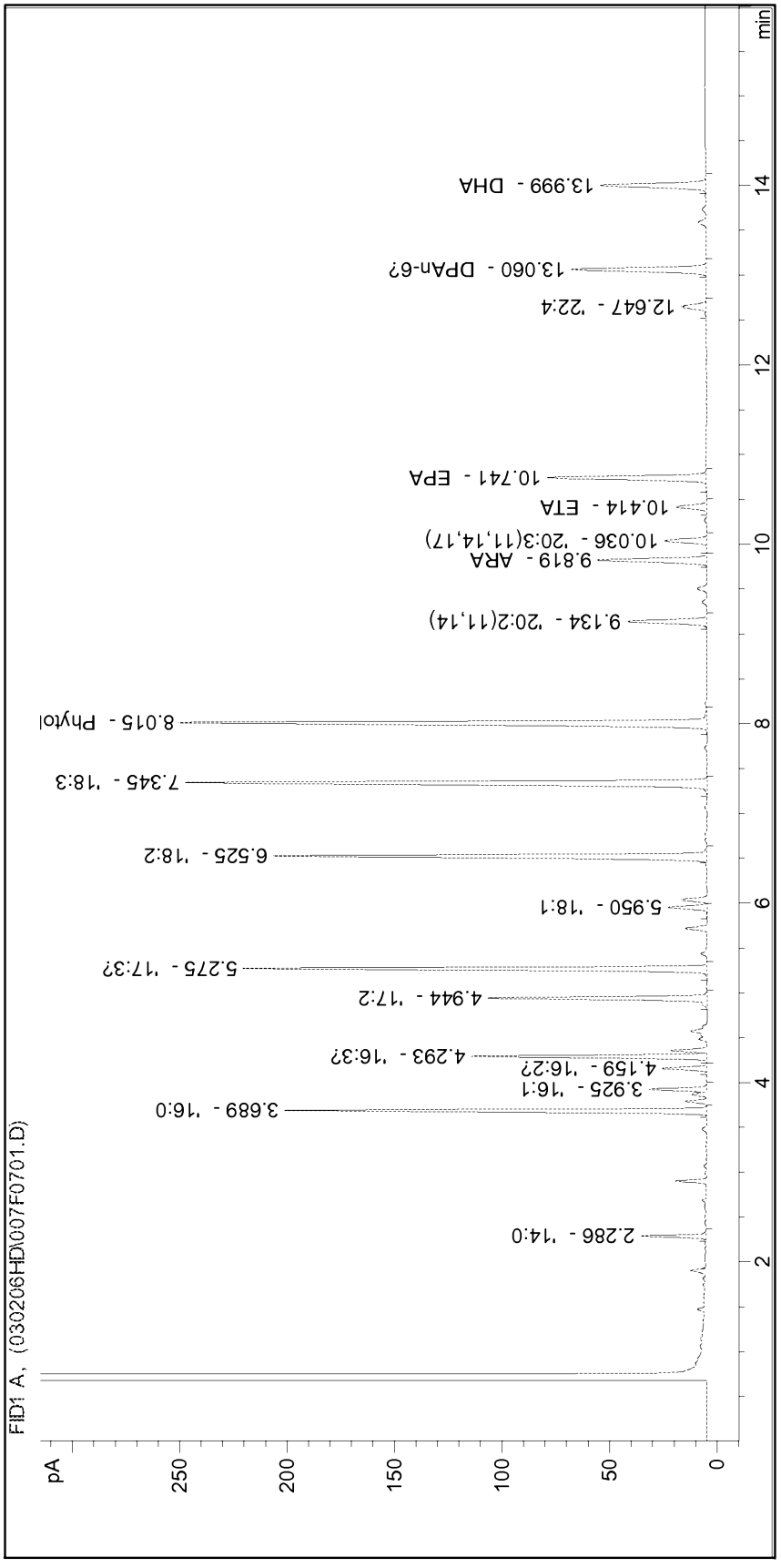
[C22:5, ω-3]
(DPA)
Docosahexaenoic Acid
[C22:6, ω-3]
(DHA)
C16/18 elongase
C14/16 elongase
Δ9 desaturase
Δ12 desaturase
Δ15 desaturase
Δ 6 desaturase
Δ 5 desaturase
Δ 4 desaturase
Δ 8 desaturase
Δ 9 elongase
C18/20 elongase
C20/22 elongase
Δ17 desaturase

FIG. 1

FIG. 2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min
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14
2.286 - '14:0
3.689 - '16:0
3.925 - '16:1
4.159 - '16:2?
4.293 - '16:3?
4.944 - '17:2
5.275 - '17:3?
5.950 - '18:1
6.525 - '18:2
7.345 - '18:3
8.015 - Phytol
9.134 - '20:2(11,14)
9.819 - ARA
10.036 - '20:3(11,14,17)
10.414 - ETA
10.741 - EPA
12.647 - '22:4
13.060 - DPAn-6?
13.999 - DHA

FIG. 3

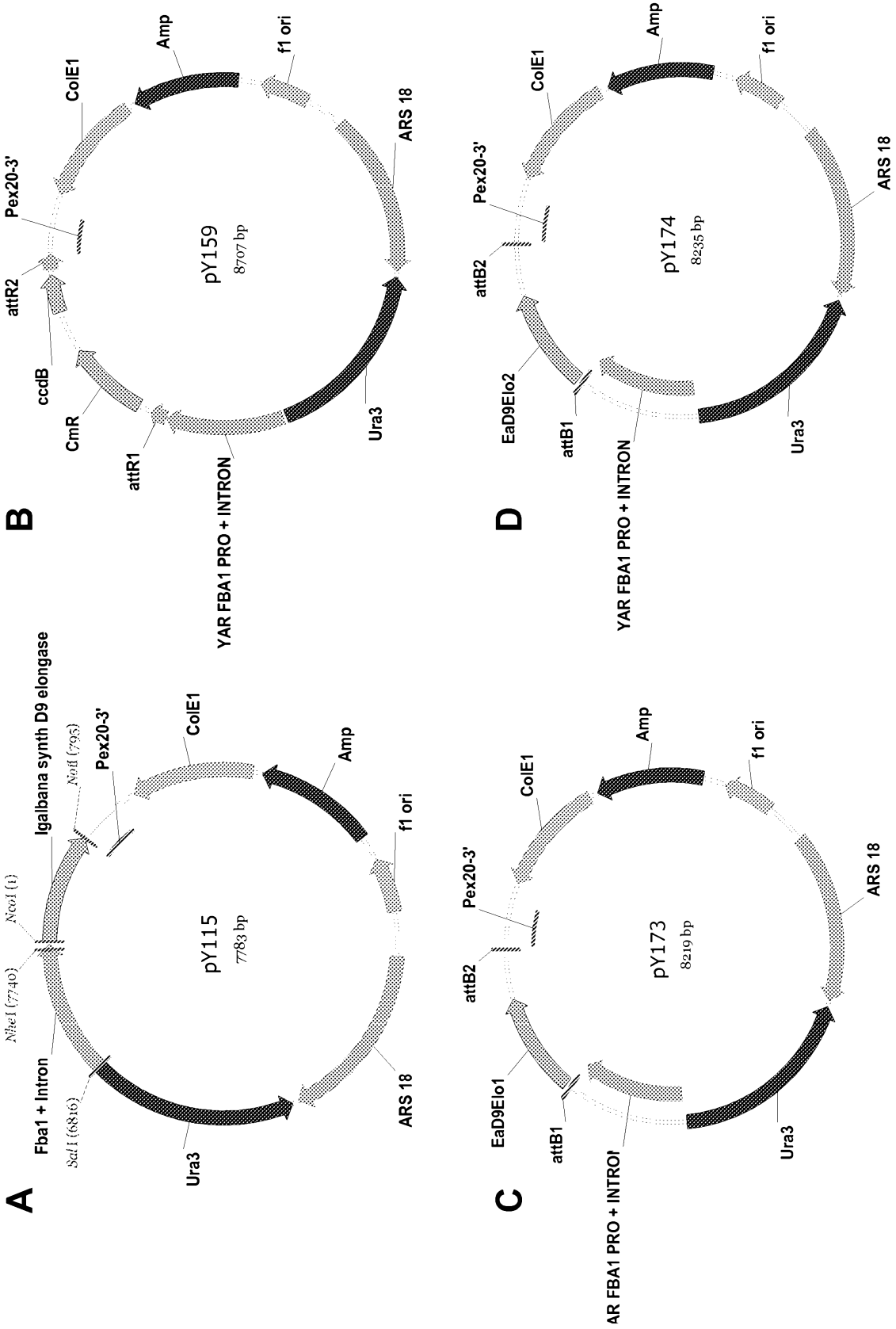

A
pY115
7783 bp
Igalbana synth D9 elongase
Fba1 + Intron
Pex20-3'
ColE1
Amp
f1 ori
Ura3
ARS 18
NheI (7740)
NcoI (1)
NotI (795)
SalI (6816)
B
pY159
8707 bp
Pex20-3'
ColE1
Amp
f1 ori
attR2
ccdB
CmR
attR1
YAR FBA1 PRO + INTRON
Ura3
ARS 18
C
pY173
8219 bp
Pex20-3'
ColE1
Amp
f1 ori
attB2
EaD9Elo1
attB1
AR FBA1 PRO + INTRON
Ura3
ARS 18
D
pY174
8235 bp
Pex20-3'
ColE1
Amp
f1 ori
attB2
EaD9Elo2
attB1
YAR FBA1 PRO + INTRON
Ura3
ARS 18

FIG. 4A

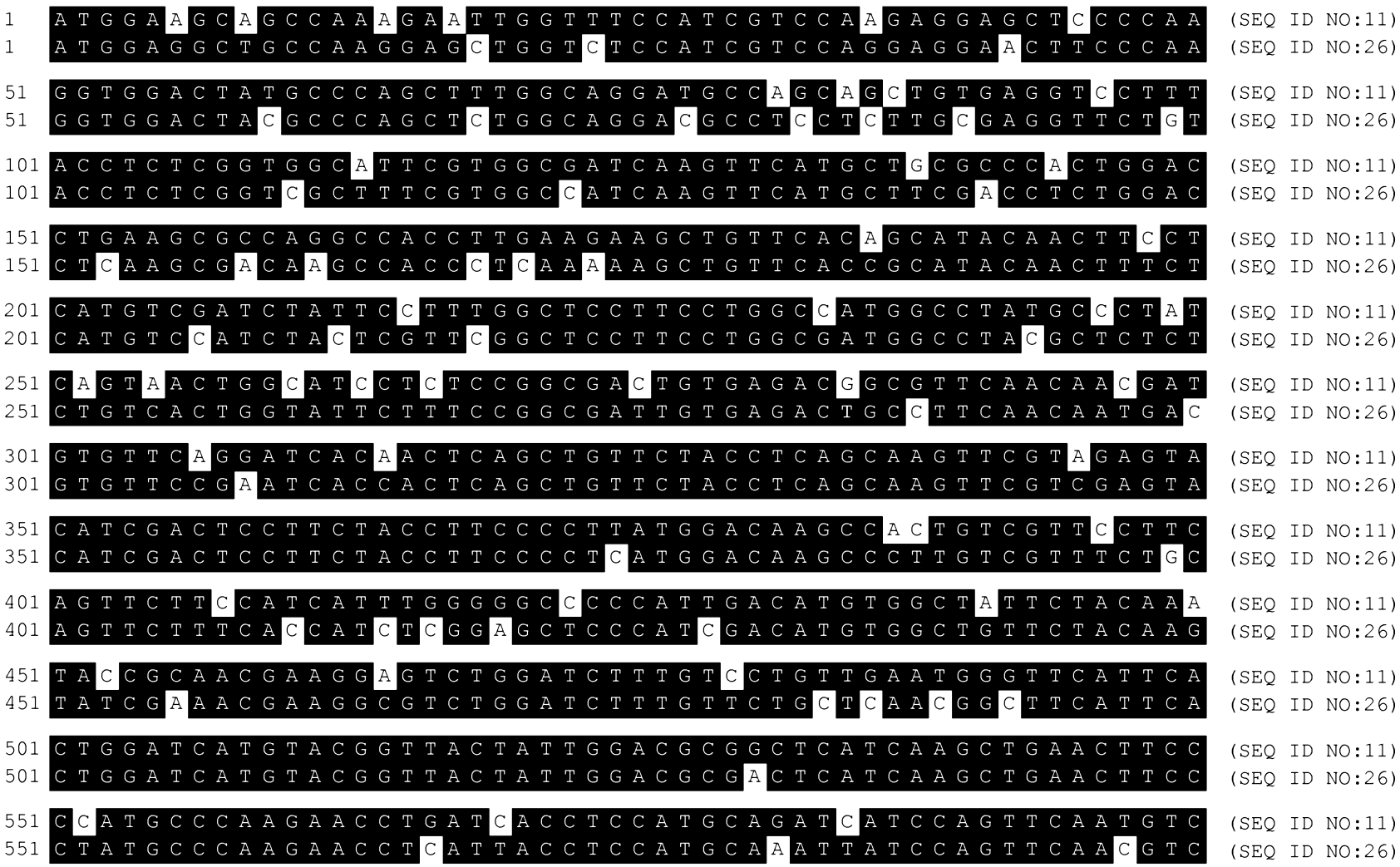

1 ATGGAAGCAGCCAAAGAATTGGTTTCCATCGTCCAAGAGGAGCTCCCAA (SEQ ID NO:11)
1 ATGGAGGCTGCCAAGGAGCTGGTCTCCATCGTCCAGGAGGAACTTCCCAA (SEQ ID NO:26)
51 GGTGGACTATGCCCAGCTTTGGCAGGATGCCAGCAGCTGTGAGGTCCTTT (SEQ ID NO:11)
51 GGTGGACTACGCCCAGCTCTGGCAGGACGCCTCCTCTTGCGAGGTTCTGT (SEQ ID NO:26)
101 ACCTCTCGGTGGCATTCGTGGCGATCAAGTTCATGCTGCGCCCACTGGAC (SEQ ID NO:11)
101 ACCTCTCGGTCGCTTTCGTGGCCATCAAGTTCATGCTTCGACCTCTGGAC (SEQ ID NO:26)
151 CTGAAGCGCCAGGCCACCTTGAAGAAGCTGTTCACAGCATACAACTTCCT (SEQ ID NO:11)
151 CTCAAGCGACAAGCCACCCTCAAAAAGCTGTTCACCGCATACAACTTTCT (SEQ ID NO:26)
201 CATGTCGATCTATTCCTTTGGCTCCTTCCTGGCCATGGCCTATGCCCTAT (SEQ ID NO:11)
201 CATGTCCATCTACTCGTTCGGCTCCTTCCTGGCGATGGCCTACGCTCTCT (SEQ ID NO:26)
251 CAGTAACTGGCATCCTCTCCGGCGACTGTGAGACGGCGTTCAACAACGAT (SEQ ID NO:11)
251 CTGTCACTGGTATTCTTTCCGGCGATTGTGAGACTGCCTTCAACAATGAC (SEQ ID NO:26)
301 GTGTTCAGGATCACAACTCAGCTGTTCTACCTCAGCAAGTTCGTAGAGTA (SEQ ID NO:11)
301 GTGTTCCGAATCACCACTCAGCTGTTCTACCTCAGCAAGTTCGTCGAGTA (SEQ ID NO:26)
351 CATCGACTCCTTCTACCTTCCCCCTTATGGACAAGCCACTGTCGTTCCTTC (SEQ ID NO:11)
351 CATCGACTCCTTCTACCTTCCCCCTCATGGACAAGCCCTTGTCGTTTCTGC (SEQ ID NO:26)
401 AGTTCTTCCATCATTTGGGGGCCCCCATTGACATGTGGCTATTCTACAAA (SEQ ID NO:11)
401 AGTTCTTTCACCATCTCGGAGCTCCCATCGACATGTGGCTGTTCTACAAG (SEQ ID NO:26)
451 TACCGCAACGAAGGAGTCTGGATCTTTGTCCTGTTGAATGGGTTCATTCA (SEQ ID NO:11)
451 TATCGAAACGAAGGCGTCTGGATCTTTGTTCTGCTCAACGGCTTCATTCA (SEQ ID NO:26)
501 CTGGATCATGTACGGTTACTATTGGACGCGGCTCATCAAGCTGAACTTCC (SEQ ID NO:11)
501 CTGGATCATGTACGGTTACTATTGGACGCGACTCATCAAGCTGAACTTCC (SEQ ID NO:26)
551 CCATGCCCAAGAACCTGATCACCTCCATGCAGATCATCCAGTTCAATGTC (SEQ ID NO:11)
551 CTATGCCCAAGAACCTCATTACCTCCATGCAAATTATCCAGTTCAACGTC (SEQ ID NO:26)

FIG. 4B

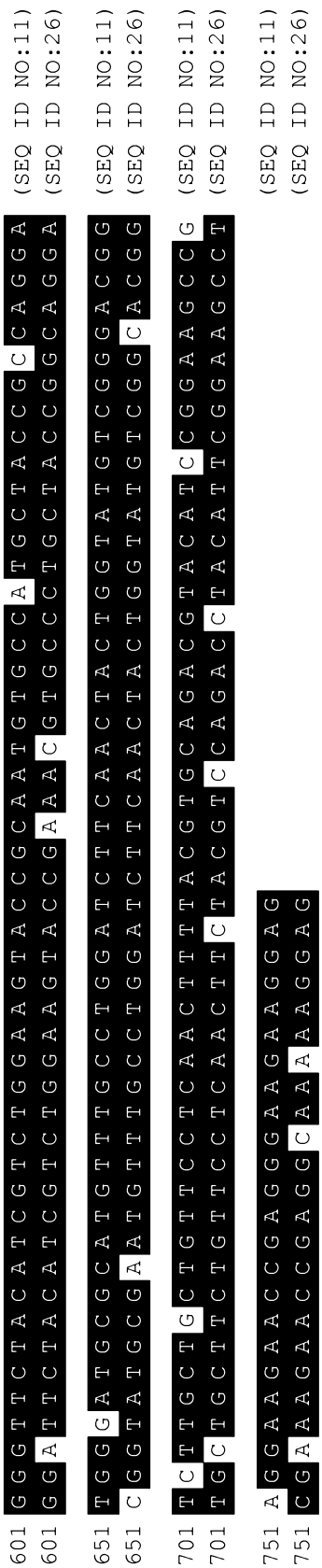
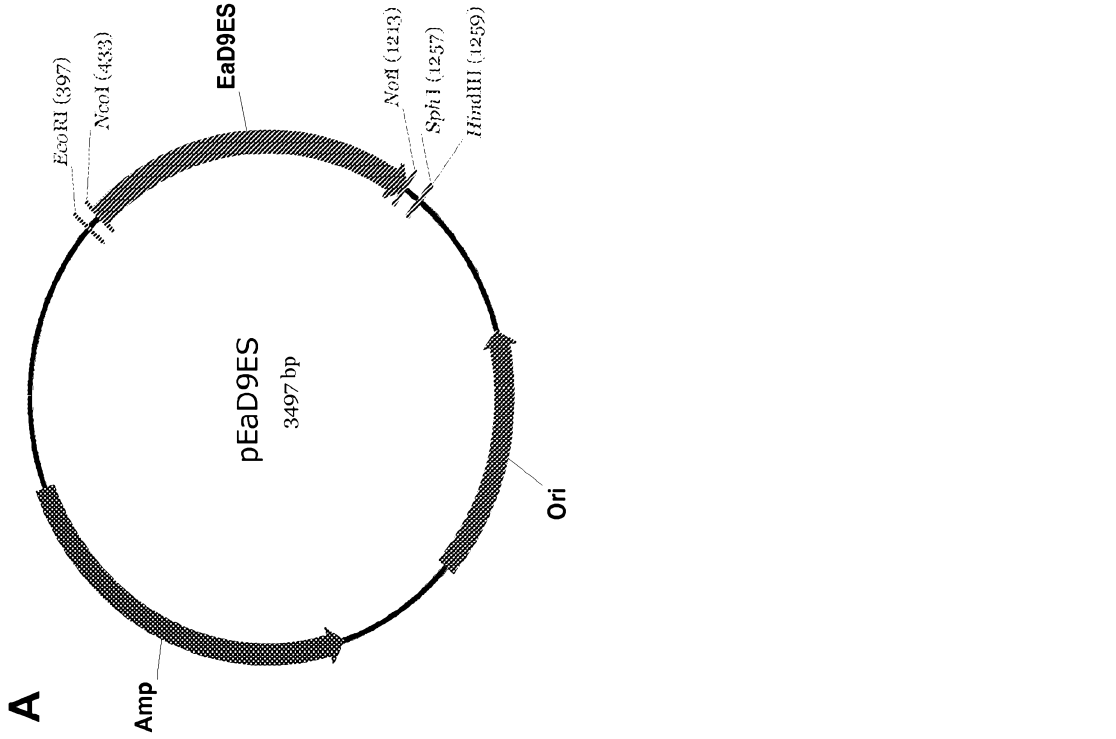

601 GGGTTCTACATCGTCTGGAAGTACCGCAATGTGCCATGCTTACCGCCAGGGA (SEQ ID NO:11)
601 GGATTCTACATCGTCTGGAAGTACCGAAACGTGCCCCTGCCTACCGGCCAGGGA (SEQ ID NO:26)
651 TGGGGATGGCGCATGTTTTGCCCTGGAATCCTTCAACTTACCTGGTATTGGTCGGGGACGGG (SEQ ID NO:11)
651 CGGTATGCCGAATGTTTGCCCTGGAATCCTTCAACTTACCTGGTATTGGTCGGCACGGG (SEQ ID NO:26)
701 TCTTGCTTGCTGTTCCTCAACTTTTACGTGCAGGACGTTACATTCCGGGAAGGCCG (SEQ ID NO:11)
701 TGCCTGCCTTCCTGTTCCTCAACTTCTACGTCCAGGACCTTACATTCGGGAAGGCCT (SEQ ID NO:26)
751 AGGGAAGGAAACCCGGAAGGGGAAGGAAGAG (SEQ ID NO:11)
751 CGAAGGAACCGAGGCAAAAAGGAG (SEQ ID NO:26)

FIG. 5


A
pEaD9ES
3497 bp
Amp
Ori
EaD9ES
EcoRI (397)
NcoI (433)
NotI (1213)
SphI (1257)
HindIII (1259)
B
pZUFmEaD9ES
7769 bp
Amp
f1 ori
ARS 18
Ura3
FBAINm
EaD9ES
Pex20-3'
ColE1
EcoRI (3114)
SphI (4460)
ClaI (4510)
PacI (4530)
PmeI (6031)
BglII (6045)
SwaI (6067)
EcoRI (6868)
NcoI (6990)
BsiWI (318)
NotI (1)