

(12) **DEMANDE DE BREVET CANADIEN**
CANADIAN PATENT APPLICATION

(13) **A1**

(86) Date de dépôt PCT/PCT Filing Date: 2008/10/03

(87) Date publication PCT/PCT Publication Date: 2009/04/09

(85) Entrée phase nationale/National Entry: 2010/03/29

(86) N° demande PCT/PCT Application No.: US 2008/078671

(87) N° publication PCT/PCT Publication No.: 2009/046248

(30) **Priorité/Priority:** 2007/10/03 (US60/977,174)

(51) Cl.Int./Int.Cl. *C12N 1/00* (2006.01),
C12N 15/00 (2006.01), *C12P 7/64* (2006.01)

(71) Demandeur/Applicant:

E.I. DU PONT DE NEMOURS AND COMPANY, US

(72) Inventeurs/Inventors:

XUE, ZHIXIONG, US;
ZHU, QUINN QUN, US;
YADAV, NARENDRA S., US;
SHARPE, PAMELA L., US;
HONG, SEUNG-PYO, US

(74) **Agent:** TORYS LLP

(54) Titre : PERTURBATIONS DES PROTEINES DES FACTEURS DE BIOGENESE DU PEROXYSOME (PEX) POUR MODIFIER LA TENEUR EN ACIDES GRAS POLYINSATURES ET LA TENEUR EN LIPIDES TOTAUX DANS DES ORGANISMES EUCARYOTES OLEAGINEUX

(54) Title: PEROXISOME BIOGENESIS FACTOR PROTEIN (PEX) DISRUPTIONS FOR ALTERING THE CONTENT OF POLYUNSATURATED FATTY ACIDS AND THE TOTAL LIPID CONTENT IN OLEAGINOUS EUKARYOTIC ORGANISMS

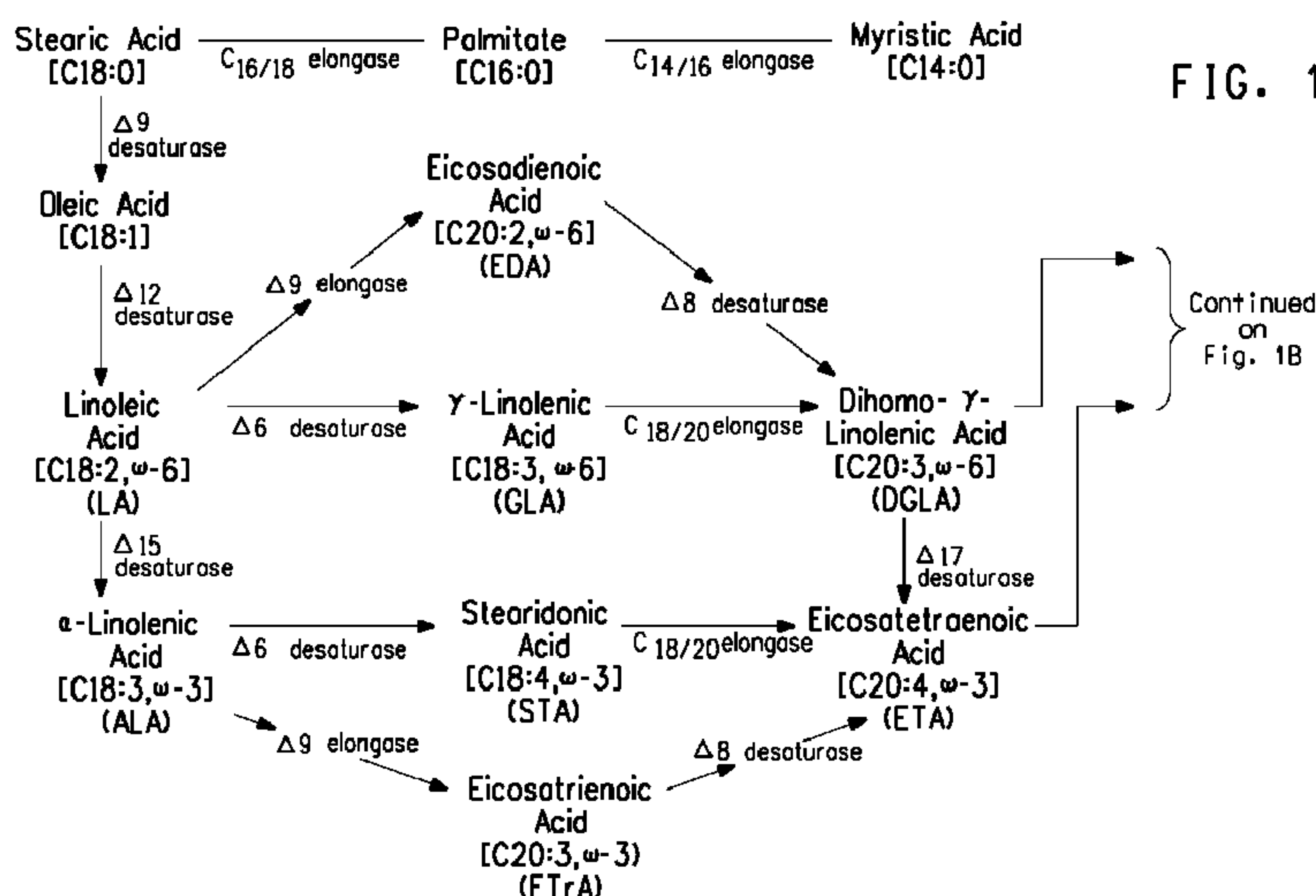


FIG. 1A

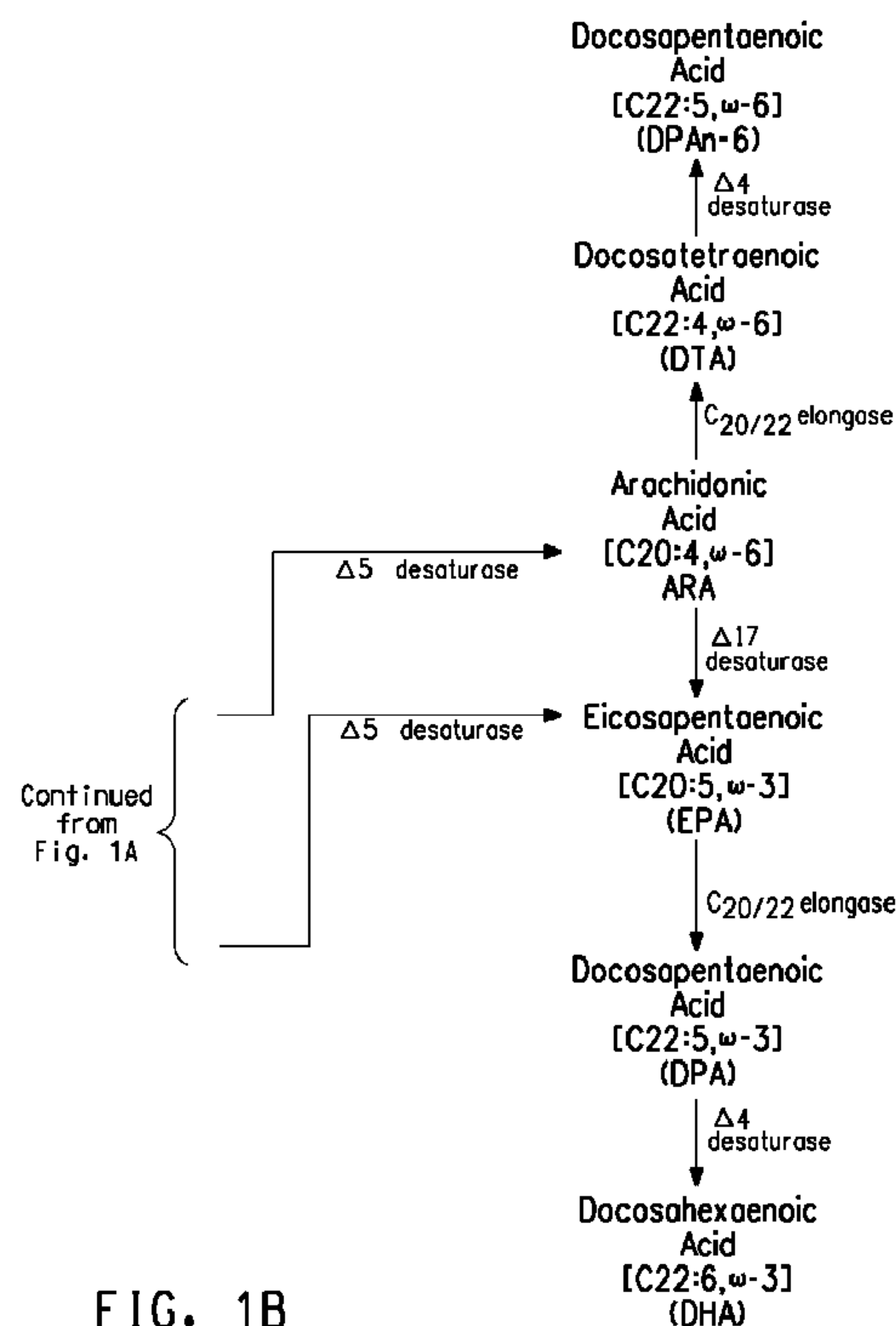


FIG. 1B

(57) Abrégé/Abstract:

Methods of increasing the amount of polyunsaturated fatty acids (PUFAs) in the total lipid fraction and in the oil fraction of PUFA-producing, oleaginous eukaryotes, accomplished by modifying the activity of peroxisome biogenesis factor (Pex) proteins. Disruptions of a chromosomal Pex3 gene, Pex10p gene or Pex16p gene in a PUFA-producing, oleaginous eukaryotic strain resulted in an increased amount of PUFAs, as a percent of total fatty acids and as a percent of dry cell weight, in the total lipid fraction and in the oil fraction of the strain, as compared to the parental strain whose native Pex protein was not disrupted.

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
9 April 2009 (09.04.2009)

PCT

(10) International Publication Number
WO 2009/046248 A1

(51) International Patent Classification:

C12N 1/00 (2006.01) *C12N 15/00* (2006.01)
C12P 7/64 (2006.01)

(21) International Application Number:

PCT/US2008/078671

(22) International Filing Date: 3 October 2008 (03.10.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/977,174 3 October 2007 (03.10.2007) US

(71) Applicant (for all designated States except US): E. I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, Delaware 19898 (US).

(72) Inventors; and

(75) Inventors/Applicants (for US only): XUE, Zhixiong [CN/US]; 111 Harvey Lane, Chadds Ford, Pennsylvania 19317-9728 (US). ZHU, Quinn, Qun [US/US]; 544 Revere Road, West Chester, Pennsylvania 19382 (US). YADAV, Narendra, S. [US/US]; 3704 Knole Lane, Chadds Ford, Pennsylvania 19317 (US). SHARPE, Pamela, L. [US/US]; 232 Cayman Court, Wilmington,

Delaware 19808 (US). HONG, Seung-Pyo [AU/US]; 9 Pierson's Ridge, Hockessin, Delaware 19707 (US).

(74) Agent: SMITH, Loretta, F.; E. I. du Pont de Nemours and Company, Legal Patent Records Center, 4417 Lancaster Pike, Wilmington, Delaware 19805 (US).

(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— with international search report

[Continued on next page]

(54) Title: PEROXISOME BIOGENESIS FACTOR PROTEIN (PEX) DISRUPTIONS FOR ALTERING THE CONTENT OF POLYUNSATURATED FATTY ACIDS AND THE TOTAL LIPID CONTENT IN OLEAGINOUS EUKARYOTIC ORGANISMS

FIG. 1A

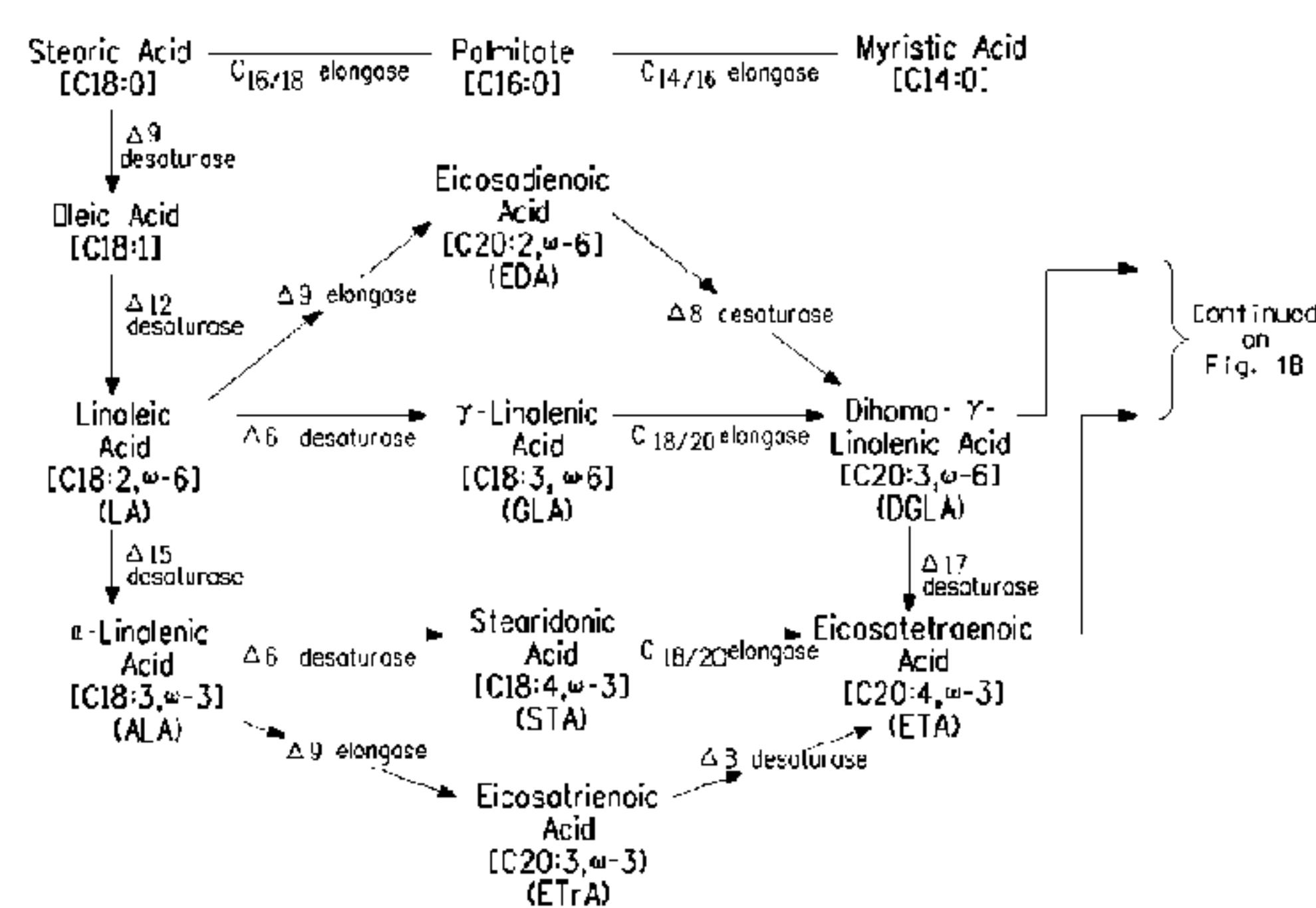
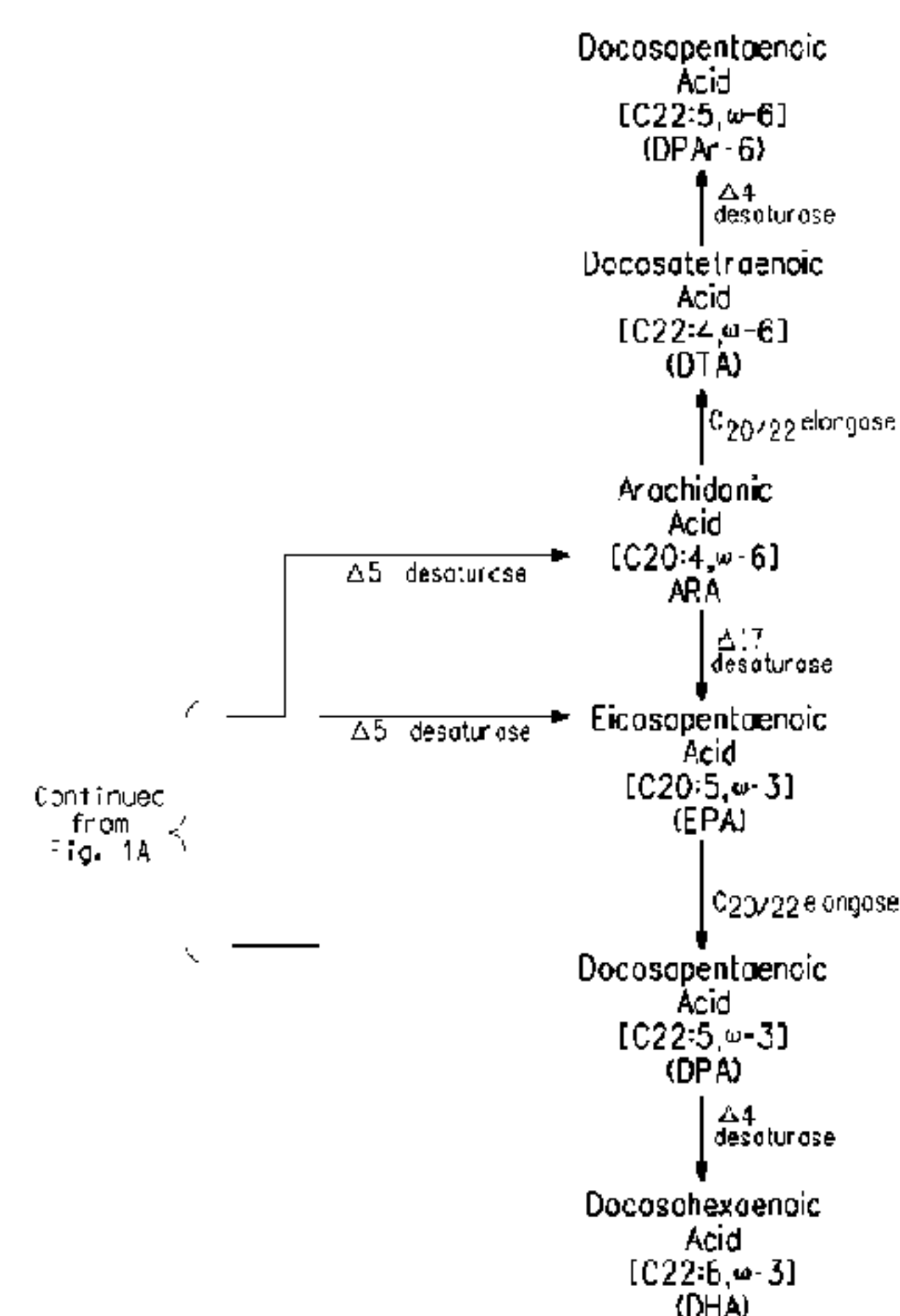


FIG. 1B



(57) Abstract: Methods of increasing the amount of polyunsaturated fatty acids (PUFAs) in the total lipid fraction and in the oil fraction of PUFA-producing, oleaginous eukaryotes, accomplished by modifying the activity of peroxisome biogenesis factor (Pex) proteins. Disruptions of a chromosomal Pex3 gene, Pex10p gene or Pex16p gene in a PUFA-producing, oleaginous eukaryotic strain resulted in an increased amount of PUFAs, as a percent of total fatty acids and as a percent of dry cell weight, in the total lipid fraction and in the oil fraction of the strain, as compared to the parental strain whose native Pex protein was not disrupted.

WO 2009/046248 A1

WO 2009/046248 A1



— *with (an) indication(s) in relation to deposited biological material furnished under Rule 13bis separately from the description*

— *with sequence listing part of description published separately in electronic form and available upon request from the International Bureau*

TITLE

PEROXISOME BIOGENESIS FACTOR PROTEIN (PEX) DISRUPTIONS FOR ALTERING THE CONTENT OF POLYUNSATURATED FATTY ACIDS AND THE TOTAL LIPID CONTENT IN OLEAGINOUS EUKARYOTIC ORGANISMS

5 This application claims the benefit of U.S. Provisional Applications No. 60/977,174 and No. 60/977,177, both filed October 3, 2007 and both hereby incorporated herein by reference in their entirety.

FIELD OF THE INVENTION

10 This invention is in the field of biotechnology. More specifically, this invention pertains to methods useful for manipulating the polyunsaturated fatty acid (PUFA) composition and lipid content of eukaryotic organisms, based on disruption of peroxisome biogenesis factor (Pex) proteins.

BACKGROUND OF THE INVENTION

15 The health benefits associated with polyunsaturated fatty acids ["PUFAs"], especially ω -3 and ω -6 PUFAs, have been well documented. In order to find ways to produce large-scale quantities of ω -3 and ω -6 PUFAs, researchers have directed their work toward the discovery of genes and the understanding of the encoded biosynthetic pathways that result in lipids and fatty acids.

20 One effort to produce these PUFAs has introduced ω -3/ ω -6 PUFA biosynthetic pathways into organisms that do not natively produce ω -3/ ω -6 PUFAs. One such organism that has been extensively manipulated is the non-oleaginous yeast, *Saccharomyces cerevisiae*. However, none of the preliminary results demonstrating limited production of linoleic acid ["LA"], γ -linolenic acid ["GLA"], α -linolenic acid ["ALA"], stearidonic acid ["STA"]
25 and/or eicosapentaenoic acid ["EPA"] are suitable for commercial exploitation.

30 Other efforts to produce large-scale quantities of ω -3/ ω -6 PUFAs have cultivated microbial organisms that natively produce the fatty acid of choice, e.g., heterotrophic diatoms *Cyclotella* sp. and *Nitzschia* sp., *Pseudomonas*, *Alteromonas* or *Shewanella* species, filamentous fungi of the genus *Pythium*, or *Mortierella elongata*, *M. exigua* or *M. hygrophila*.

 All these efforts suffer from an inability to substantially improve the

yield of oil or to control the characteristics of the oil composition produced, since the fermentations rely on the natural abilities of the microbes themselves.

Commonly owned U.S. Pat. No. 7,238,482 describes the use of the
5 oleaginous yeast *Yarrowia lipolytica* as a production host for the production of PUFAs. Oleaginous yeast are defined as those yeast that are naturally capable of oil synthesis and accumulation, where greater than 25% of the cellular dry weight is typical. Optimization of the production host has been described in the art (see for example Int'l. App.
10 Pub. No. WO 2006/033723, U.S. Pat. App. Pub. No. 2006-0094092, U.S. Pat. App. Pub. No. 2006-0115881, and U.S. Pat. App. Pub. No. 2006-0110806). The recombinant strains described therein comprise various chimeric genes expressing multiple copies of heterologous desaturases, elongases and acyltransferases and optionally comprise various native
15 desaturase and acyltransferase knockouts to enable PUFA synthesis and accumulation. Further optimization of the host cell is needed for commercial production of PUFAs.

Lin Y. et al suggest that peroxisomes are required for both catabolic and anabolic lipid metabolism (*Plant Physiology*, 135:814-827 (2004)).
20 However, this hypothesis was based on studies with a homolog of Pex16p in *Arabidopsis* mutants that had both abnormal peroxisome biogenesis and fatty acid synthesis (i.e., a reduction of oil to approximately 10-16% of wild type in *sse1* seeds was reported). Binns, D. et al. (*J. Cell Biol.*, 173(5):719-731 (2006)) also document an intimate collaboration between
25 peroxisomes and lipid bodies in *Saccharomyces cerevisiae*. But, previous studies of Pex knockouts have not been performed in a PUFA-producing organism.

Applicants have solved the stated problem of optimizing host cells for commercial production of PUFAs by the unpredictable mechanism of
30 disruption of peroxisome biogenesis factor proteins in a PUFA-producing organism, which leads to the unpredictable result of an increase in the amount of PUFAs, as a percent of total fatty acids, in a recombinant

PUFA-producing strain of *Y. lipolytica*. Novel strains containing disruptions in peroxisome biogenesis factor proteins are described herein.

SUMMARY OF THE INVENTION

Described herein are methods of increasing the weight percent of at least one polyunsaturated fatty acid ["PUFA"] relative to the weight percent of total fatty acids ["TFAs"] in an oleaginous eukaryotic organism having a total lipid content, a total lipid fraction and an oil fraction, comprising:

a) providing an oleaginous eukaryotic organism comprising:

- 1) genes encoding a functional polyunsaturated fatty acid biosynthetic pathway; and
 - 2) a disruption in a native gene encoding a peroxisome biogenesis factor protein, thereby providing a PEX-disrupted organism, and
- b) growing the PEX-disrupted organism under conditions as to increase the weight percent of at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in the total lipid fraction or in the oil fraction, when compared to the weight percent of the at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in the total lipid fraction or in the oil fraction in the oleaginous eukaryotic organism in which no native gene encoding a peroxisome biogenesis factor protein has been disrupted.

This method of increasing may also be used to increase the percent of at least one polyunsaturated fatty acid ["PUFA"] relative to the dry cell weight ["DCW"] by applying the same steps (a) and (b) .

In some of the methods described here, the weight percent of the PUFA relative to the weight percent of the TFAs is increased at least 1.3 fold.

In some of the described methods, the total lipid content in the PEX-disrupted organism may be increased or decreased compared with that of an oleaginous eukaryote having no disruption in a native PEX gene.

In any of these methods, the increased PUFA may be a single PUFA or a combination of PUFAs. In either case, the increased PUFA or

increased combination of PUFAs can include linoleic acid, conjugated linoleic acid, γ -linolenic acid, dihomo- γ -linolenic acid, arachidonic acid, docosatetraenoic acid, ω -6 docosapentaenoic acid, α -linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, ω -3 docosapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, docosaheptaenoic acid, hydroxylated or epoxy fatty acids of these, a C_{18} polyunsaturated fatty acid or a combination of these, a C_{20} polyunsaturated fatty acid or a combination of these, a combination of C_{20} -22 polyunsaturated fatty acids and a C_{22} polyunsaturated fatty acid or a combination of these.

In any of these methods, the PEX-disrupted organism may be a member of the following: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon*, *Lipomyces*, *Mortierella*, *Thraustochytrium*, *Schizochytrium*, and *Saccharomyces* having the property of oleaginity. And, in any of the described methods, the PUFA biosynthetic pathway includes genes that encodes any or a combination of the following enzymes: $\Delta 9$ desaturase, $\Delta 12$ desaturase, $\Delta 6$ desaturase, $\Delta 5$ desaturase, $\Delta 17$ desaturase, $\Delta 8$ desaturase, $\Delta 15$ desaturase, $\Delta 4$ desaturase, $C_{14/16}$ elongase, $C_{16/18}$ elongase, $C_{18/20}$ elongase, $C_{20/22}$ elongase and $\Delta 9$ elongase.

The disruption may occur in a PEX gene that encodes a peroxisome biogenesis factor protein that includes the following: Pex1p, Pex 2p, Pex3p, Pex3Bp, Pex4p, Pex5p, Pex5Bp, Pex5Cp, Pex5/20p, Pex6p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex15p, Pex16p, Pex17p, Pex14/17p, Pex18p, Pex19p, Pex20p, Pex21p, Pex21Bp, Pex22p, Pex22p-like and Pex26p. And in any of these methods, the disruption may be a gene knockout or a deletion in a portion of the gene that encodes the C-terminal portion of the protein. In some of these methods, the deletion is in the portion of the gene encoding the C-terminal portion of the C_3HC_4 zinc ring finger motif of the protein.

Also described herein is the oil fraction or the total lipid fraction in a PEX-disrupted organism, which has experienced an increase in the weight percent of at least one PUFA accomplished by the method of Claim 1.

Described herein is also a PEX-disrupted *Yarrowia lipolytica*, having a disruption in a native gene encoding Pex3p or Pex10p or Pex16p. This *Y. lipolytica* may have ATCC designation ATCC PTA-8614 (strain Y4128).

5

BIOLOGICAL DEPOSITS

The following biological materials have been deposited with the American Type Culture Collection (ATCC), 10801 University Boulevard, Manassas, VA 20110-2209, and bear the following designations, accession numbers and dates of deposit.

Biological Material	Accession No.	Date of Deposit
<i>Yarrowia lipolytica</i> Y2047	ATCC PTA-7186	October 26, 2005
<i>Yarrowia lipolytica</i> Y2201	ATCC PTA-7185	October 26, 2005
<i>Yarrowia lipolytica</i> Y2096	ATCC PTA-7184	October 26, 2005
<i>Yarrowia lipolytica</i> Y3000	ATCC PTA-7187	October 26, 2005
<i>Yarrowia lipolytica</i> Y4128	ATCC PTA-8614	August 23, 2007
<i>Yarrowia lipolytica</i> Y4127	ATCC PTA-8802	November 29, 2007

10

The biological materials listed above were deposited under the terms of the Budapest Treaty on the International Recognition of the Deposit of Microorganisms for the Purposes of Patent Procedure. The listed deposit will be maintained in the indicated international depository for at least 30 years and will be made available to the public upon the grant of a patent disclosing it. The availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by government action.

15

BRIEF DESCRIPTION OF THE DRAWINGS AND

20

SEQUENCE LISTINGS

FIG. 1 consists of FIG. 1A and FIG 1B, which together illustrate the ω -3/ ω -6 fatty acid biosynthetic pathway, and should be viewed together when considering the description of this pathway below.

25

FIG. 2A provides an alignment of the C₃HC₄ zinc ring finger motifs of the *Yarrowia lipolytica* Pex10p (i.e., amino acids 327-364 of SEQ ID NO:10 [GenBank Accession No. CAG81606]), the *Yarrowia lipolytica* Pex2p (i.e., amino acids 266-323 of SEQ ID NO:2 [GenBank Accession No. CAG77647]) and the *Yarrowia lipolytica* Pex12p (i.e., amino acids

342-391 of SEQ ID NO:11 [GenBank Accession No. CAG81532]), with cysteine and histidine residues of the conserved C₃HC₄ zinc ring finger motif indicated by astericks.

FIG. 2B schematically illustrates the proposed interaction between various amino acid residues of the *Y. lipolytica* Pex10p C₃HC₄ finger motif and the two zinc ions to which they bind.

FIG. 3A diagrams the development of *Yarrowia lipolytica* strain Y4128, producing 37.6% EPA in the total lipid fraction.

FIG. 3B provides a plasmid map for pZP3-Pa777U.

FIG. 4 provides plasmid maps for the following: (A) pY117; and, (B) pZP2-2988.

FIG. 5 provides plasmid maps for the following: (A) pZKUE3S; and, (B) pFBAIN-MOD-1.

FIG. 6 provides plasmid maps for the following: (A) pFBAIN-PEX10; and, (B) pEXP-MOD-1.

FIG. 7A provides a plasmid map for pPEX10-1. FIG. 7B diagrams the development of *Yarrowia lipolytica* strain Y4184U.

FIG. 8 provides plasmid maps for the following: (A) pZKL1-2SP98C; and, (B) pZKL2-5U89GC.

FIG. 9 provides plasmid maps for the following: (A) pYPS161; and, (B) pYRH13.

FIG. 10 diagrams the development of *Yarrowia lipolytica* strain Y4305U3.

FIG. 11 provides plasmid maps for the following: (A) pZKUM; and, (B) pZKD2-5U89A2.

FIG. 12 provides plasmid maps for the following: (A) pY87; and, (B) pY157.

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
5 and amino acid sequence data comply with the rules set forth in 37 C.F.R. §1.822.

SEQ ID NOs:1-86 are primers, ORFs encoding genes or proteins (or portions thereof), or plasmids, as identified in Table 1.

10

Table 1

Summary Of Nucleic Acid And Protein SEQ ID Numbers

Description and Abbreviation	Nucleic acid SEQ ID NO.	Protein SEQ ID NO.
<i>Yarrowia lipolytica</i> Pex1p (GenBank Accession No. CAG82178)	--	1 (1024 AA)
<i>Yarrowia lipolytica</i> Pex2p (GenBank Accession No. CAG77647)	--	2 (381 AA)
<i>Yarrowia lipolytica</i> Pex3p (GenBank Accession No. CAG78565)	--	3 (431 AA)
<i>Yarrowia lipolytica</i> Pex3Bp (GenBank Accession No. CAG83356)	--	4 (395 AA)
<i>Yarrowia lipolytica</i> Pex4p (GenBank Accession No. CAG79130)	--	5 (153 AA)
<i>Yarrowia lipolytica</i> Pex5p (GenBank Accession No. CAG78803)	--	6 (598 AA)
<i>Yarrowia lipolytica</i> Pex6p (GenBank Accession No. CAG82306)	--	7 (1024 AA)
<i>Yarrowia lipolytica</i> Pex7p (GenBank Accession No. CAG78389)	--	8 (356 AA)
<i>Yarrowia lipolytica</i> Pex8p (GenBank Accession No. CAG80447)	--	9 (671 AA)
<i>Yarrowia lipolytica</i> Pex10p (GenBank Accession No. CAG81606)	--	10 (377 AA)
<i>Yarrowia lipolytica</i> Pex12p (GenBank Accession No. CAG81532)	--	11 (408 AA)
<i>Yarrowia lipolytica</i> Pex13p (GenBank Accession No. CAG81789)	--	12 (412 AA)
<i>Yarrowia lipolytica</i> Pex14p (GenBank Accession No. CAG79323)	--	13 (380 AA)
<i>Yarrowia lipolytica</i> Pex16p (GenBank Accession No. CAG79622)	--	14 (391 AA)
<i>Yarrowia lipolytica</i> Pex17p (GenBank Accession No. CAG84025)	--	15 (225 AA)
<i>Yarrowia lipolytica</i> Pex19p (GenBank Accession No. AAK84827)	--	16 (324 AA)
<i>Yarrowia lipolytica</i> Pex20p (GenBank	--	17

Accession No. CAG79226)		(417 AA)
<i>Yarrowia lipolytica</i> Pex22p (GenBank Accession No. CAG77876)	--	18 (195 AA)
<i>Yarrowia lipolytica</i> Pex26p (GenBank Accession No. NC_006072, antisense translation of nucleotides 117230-118387)	--	19 (386 AA)
Contig comprising <i>Yarrowia lipolytica</i> Pex10 gene encoding peroxisomal biogenesis factor protein (Pex10p) (GenBank Accession No. AB036770)	20 (3387 bp)	--
<i>Yarrowia lipolytica</i> Pex10 (GenBank Accession No. AB036770, nucleotides 1038- 2171) (the protein sequence is 100% identical to SEQ ID NO:10)	21 (1134 bp)	22 (377 AA)
<i>Yarrowia lipolytica</i> Pex10 (GenBank Accession No. AJ012084, which corresponds to nucleotides 1107-2171 of GenBank Accession No. AB036770) (the first 23 amino acids are truncated with respect to the protein sequences of SEQ ID NOs:10 and 22)	23 (1065 bp)	24 (354 AA)
<i>Yarrowia lipolytica</i> Pex10p C ₃ HC ₄ zinc ring finger motif (i.e., amino acids 327-364 of SEQ ID NO:10)	--	25 (38 AA)
<i>Yarrowia lipolytica</i> truncated Pex10p (GenBank Accession No. CAG81606 [SEQ ID NO:10], with C-terminal 32 amino acid deletion)	--	26 (345 AA)
<i>Yarrowia lipolytica</i> mutant acetohydroxyacid synthase (AHAS) gene comprising a W497L mutation	27 (2987 bp)	--
Plasmid pZP3-Pa777U	28 (13,066 bp)	--
Plasmid pY117	29 (9570 bp)	--
Plasmid pZP2-2988	30 (15,743 bp)	--
Plasmid pZKUE3S	31 (6303 bp)	--
Primer pZP-GW-5-1	32	--
Primer pZP-GW-5-2	33	--
Primer pZP-GW-5-3	34	--
Primer pZP-GW-5-4	35	--
Primer pZP-GW-3-1	36	--
Primer pZP-GW-3-2	37	--
Primer pZP-GW-3-3	38	--
Primer pZP-GW-3-4	39	--
Genome Walker adaptor [top strand]	40	--
Genome Walker adaptor [bottom strand]	41	--
Nested adaptor primer	42	--
Primer Per10 F1	43	--
Primer ZPGW-5-5	44	--
Primer Per10 R	45	--
Plasmid pFBAIN-MOD-1	46	--

	(7222 bp)	
Plasmid pFBAIn-PEX10	47 (8133 bp)	--
Primer PEX10-R-BsiWI	48	--
Primer PEX10-F1-Sall	49	--
Primer PEX10-F2-Sall	50	--
Plasmid pEXP-MOD1	51 (7277 bp)	--
Plasmid pPEX10-1	52 (7559 bp)	--
Plasmid pPEX10-2	53 (8051 bp)	--
Plasmid pZKL1-2SP98C	54 (15,877 bp)	--
Plasmid pZKL2-5U89GC	55 (15,812 bp)	--
Plasmid pYPS161	56 (7966 bp)	--
Primer Pex-10del1 3'.Forward	57	--
Primer Pex-10del2 5'.Reverse	58	--
Plasmid pYRH13	59 (8673 bp)	--
Primer PEX16Fii	60	--
Primer PEX16Rii	61	--
Primer 3UTR-URA3	62	--
Primer Pex16-conf	63	--
Real time PCR primer ef-324F	64	--
Real time PCR primer ef-392R	65	--
Real time PCR primer Pex16-741F	66	--
Real time PCR primer Pex16-802R	67	--
Nucleotide portion of TaqMan probe ef-345T	68	--
Nucleotide portion of TaqMan probe PEX16-760T	69	--
Plasmid pZKUM	70 (4313 bp)	--
Plasmid pZKD2-5U89A2	71 (15,966 bp)	--
<i>Yarrowia lipolytica</i> diacylglycerol acyltransferase (DGAT2) (U.S. Patent 7,267,976)	72 (2119 bp)	73 (514 AA)
Synthetic $\Delta 12$ desaturase derived from <i>Fusarium moniliforme</i> , codon-optimized for expression in <i>Yarrowia lipolytica</i> ("FmD12S")	74 (1434 bp)	75 (477 AA)
Synthetic mutant $\Delta 8$ desaturase ("EgD8M"), derived from <i>Euglena gracilis</i> ("EgD8S"; U.S. Patent 7,256,033)	76 (1272 bp)	77 (422 AA)
Synthetic $\Delta 9$ elongase derived from <i>Eutreptiella</i> sp. CCMP389 codon-optimized for expression in <i>Yarrowia lipolytica</i> ("E389D9eS")	78 (792 bp)	79 (263 AA)
Synthetic $\Delta 5$ desaturase derived from <i>Euglena gracilis</i> , codon-optimized for expression in <i>Yarrowia lipolytica</i> ("EgD5S")	80 (1350 bp)	81 (449 AA)

Plasmid pY157	82 (6356 bp)	--
Plasmid pY87	83 (5910 bp)	--
<i>Escherichia coli</i> LoxP recombination site, recognized by a Cre recombinase enzyme	84 (34 bp)	--
Primer UP 768	85	--
Primer LP 769	86	--

DETAILED DESCRIPTION OF THE INVENTION

Described herein are generalized methods to manipulate the concentration (as a percent of total fatty acids) and content (as a percent of the dry cell weight) of long-chain polyunsaturated fatty acids [“LC-PUFAs”] in PUFA-producing eukaryotic organisms. These methods rely on disruption of a native peroxisome biogenesis factor [“Pex”] protein within the host and will have wide-spread applicability to a variety of eukaryotic organisms having native or genetically-engineered ability to produce PUFAs, including algae, fungi, oomycetes, yeast, euglenoids, stramenopiles, plants and some mammalian systems.

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. For example, PUFAs may be incorporated into cooking oils, fats or margarines and ingested as part of a consumer's typical diet, thereby giving the consumer desired dietary supplementation. Further, 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, either human or veterinary.

Definitions

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

“Open reading frame” is abbreviated as “ORF”.

“Polymerase chain reaction” is abbreviated as “PCR”.

“American Type Culture Collection” is abbreviated as “ATCC”.

“Polyunsaturated fatty acid(s)” is abbreviated as “PUFA(s)”.

“Triacylglycerols” are abbreviated as “TAGs”.

“Total fatty acids” are abbreviated as “TFAs”.

“Fatty acid methyl esters” are abbreviated as “FAMES”.

“Dry cell weight” is abbreviated as “DCW”.

5 The term “invention” or “present invention” as used herein is not meant to be limiting but applies generally to any of the inventions defined in the claims or described herein.

10 The term “peroxisomes” refers to ubiquitous organelles found in all eukaryotic cells. They have a single lipid bilayer membrane that separates their contents from the cytosol and that contains various membrane proteins essential to the functions described below. Peroxisomes selectively import proteins via an “extended shuttle mechanism”. More specifically, there are at least 32 known peroxisomal proteins, also known as peroxins, which participate in the process of importing proteins by means of ATP hydrolysis through the peroxisomal membrane. Some peroxins comprise a specific protein signal, i.e., a peroxisomal targeting signal or “PTS”, at either the N-terminus or C-terminus to signal that importation through the peroxisomal membrane should occur. Once cellular proteins are imported into the peroxisome, they are typically subjected to some means of degradation. For example, peroxisomes contain oxidative enzymes, such as catalase, D-amino acid oxidase and uric acid oxidase, that enable degradation of substances that are toxic to the cell. Alternatively, peroxisomes breakdown fatty acid molecules to produce free molecules of acetyl-CoA which are exported back to the cytosol, in a process called β -oxidation.

25 The terms “peroxisome biogenesis factor protein”, “peroxin” and “Pex protein” are interchangeable and refer to proteins involved in peroxisome biogenesis and/or that participate in the process of importing cellular proteins by means of ATP hydrolysis through the peroxisomal membrane. The acronym of a gene that encodes any of these proteins is “Pex gene”. A system for nomenclature of Pex genes is described by Distel et al., *J. Cell Biol.*, 135:1-3 (1996). At least 32 different Pex genes have been identified so far in various eukaryotic organisms. Many Pex

genes have been isolated from the analysis of mutants that demonstrated abnormal peroxisomal functions or structures. Based on a review by Kiel, J. A. K. W., et al. (*Traffic*, 7:1291-1303 (2006)), wherein *in silico* analysis of the genomic sequences of 17 different fungal species was performed, the following Pex proteins were identified: Pex1p, Pex2p, Pex3p, Pex3Bp, Pex4p, Pex5p, Pex5Bp, Pex5Cp, Pex5/20p, Pex6p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex15p, Pex16p, Pex17p, Pex14/17p, Pex18p, Pex19p, Pex20p, Pex21p, Pex21Bp, Pex22p, Pex22p-like and Pex26p. Thus, each of these proteins is referred to herein as a "Pex protein", a "peroxin" or a "peroxisome biogenesis factor protein", and is encoded by at least one "Pex gene".

The term "conserved domain" or "motif" refers to 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. Of relevance herein, Pex2p, Pex10p and Pex12p all share a cysteine-rich motif near their carboxyl termini, known as a C₃HC₄ zinc ring finger motif. This motif appears to be required for their activities, involved in protein docking and translocation into the peroxisome (Kiel, J. A. K. W., et al., *Traffic*, 7:1291-1303 (2006)).

The term "C₃HC₄ zinc ring finger motif" or "C₃HC₄ motif" generically refers to a conserved cysteine-rich motif that binds two zinc ions, identified by the presence of a sequence of amino acids as set forth in Formula I:

Formula I: CX₂CX₉₋₂₇CX₁₋₃HX₂CX₂CX₄₋₄₈CX₂C

The C₃HC₄ zinc ring finger motif within the *Yarrowia lipolytica* gene encoding the peroxisome biogenesis factor 10 protein, i.e., YIPex10p, is

located between amino acids 327-364 of SEQ ID NO:10 and is defined by a $CX_2CX_{11}CX_1HX_2CX_2CX_{10}CX_2C$ motif (SEQ ID NO:25). The C_3HC_4 zinc ring finger motif within the *Y. lipolytica* gene encoding the peroxisome biogenesis factor 2 protein, i.e., YIPex2p, is located between amino acids
5 266-323 of SEQ ID NO:2. The *Y. lipolytica* peroxisome biogenesis factor 12 protein, i.e., YIPex12p, contains an imperfect C_3HC_4 ring-finger motif located between amino acids 342-391 of SEQ ID NO:11. The protein sequences corresponding to the C_3HC_4 zinc ring finger motif of YIPex10, YIPex2 and YIPex12 are aligned in FIG. 2A; asterisks denote the
10 conserved cysteine or histidine residues of the motif.

YIPex10, YIPex2 and YIPex12 are thought to form a ring finger complex by protein-protein interaction. The proposed interaction between the cystine and histidine residues of the YIPex10p C_3HC_4 finger motif with two zinc residues is schematically diagrammed in FIG. 2B.

15 The term "Pex10" refers to the gene encoding the peroxisome biogenesis factor 10 protein or peroxisomal assembly protein Peroxin 10, wherein the peroxin protein is hereinafter referred to as "Pex10p". The function of Pex10p has not been clearly elucidated, although studies in other organisms have revealed that Pex10 products are localized in the
20 peroxisomal membrane and are essential to the normal functioning of the organelle. A C_3HC_4 zinc ring finger motif appears to be conserved in the C-terminal region of Pex10p (Kalish, J. E. et al., *Mol. Cell Biol.*, 15:6406-6419 (1995); Tan, X. et al., *J. Cell Biol.*, 128:307-319 (1995); Warren, D. S., et al., *Am. J. Hum. Genet.*, 63:347-359 (1998)) and is required for
25 enzymatic activity.

The term "YIPex10" refers to the *Yarrowia lipolytica* gene encoding the peroxisome biogenesis factor 10 protein, wherein the protein is hereinafter referred to as "YIPex10p". This particular peroxin was recently studied by Sumita et al. (*FEMS Microbiol. Lett.*, 214:31-38 (2002)). The
30 nucleotide sequence of YIPex10 was registered in GenBank under multiple accession numbers, including GenBank Accession No. CAG81606 (SEQ ID NO:10), No. AB036770 (SEQ ID NOs:20, 21 and 22) and No. AJ012084 (SEQ ID NOs:23 and 24). The YIPex10p sequence set

forth in SEQ ID NO:24 is 354 amino acids in length. In contrast, the YIPex10p sequences set forth in SEQ ID NO:10 and SEQ ID NO:22 are each 377 amino acids in length, as the 100% identical sequences possess an additional 23 amino acids at the N-terminus of the protein

5 (corresponding to a different start codon than that identified in GenBank Accession No. AJ012084 (SEQ ID NO:24)).

The term “Pex3” refers to the gene encoding the peroxisome biogenesis factor 3 protein or peroxisomal assembly protein Peroxin 3, wherein the peroxin protein is hereinafter referred to as “Pex3p”. Although
10 mechanistic details concerning the function of Pex3p have not been clearly resolved, it is clear that Pex3p is a peroxisomal integral membrane protein required early in peroxisome biogenesis for formation of the peroxisomal membrane (see, e.g., Baerends, R. J. et al., *J. Biol. Chem.*, 271:8887-8894 (1996); Bascom, R.A. et al, *Mol. Biol. Cell*, 14:939-957
15 (2003)).

The term “YIPex3” refers to the *Yarrowia lipolytica* gene encoding the peroxisome biogenesis factor 3 protein, wherein the protein is hereinafter referred to as “YIPex3p”. The nucleotide sequence of YIPex3 was registered in GenBank as Accession No. CAG78565 (SEQ ID NO:3).

20 The term “Pex16” refers to the gene encoding the peroxisome biogenesis factor 16 protein or peroxisomal assembly protein Peroxin 16, wherein the peroxin protein is hereinafter referred to as “Pex16p”. The function of Pex16p has not been clearly elucidated, although studies in various organisms have revealed that Pex16 products play a role in the
25 formation of the peroxisomal membrane and regulation of peroxisomal proliferation (Platta, H.W. and R. Erdmann, *Trends Cell Biol.*, 17(10):474-484 (2007)).

The term “YIPex16” refers to the *Yarrowia lipolytica* gene encoding the peroxisome biogenesis factor 16 protein, wherein the protein is
30 hereinafter referred to as “YIPex16p”. This particular peroxin was described by Elizen G. A., et al. (*J. Cell Biol.*, 137:1265-1278 (1997)) and Titorenko, V. I. et al. (*Mol. Cell Biol.*, 17:5210-5226 (1997)). The

nucleotide sequence of YIPex16 was registered in GenBank as Accession No. CAG79622 (SEQ ID NO:14).

The term "disruption" in or in connection with a native Pex gene refers to an insertion, deletion, or targeted mutation within a portion of that gene, that results in either a complete gene knockout such that the gene is deleted from the genome and no protein is translated or a translated Pex protein having an insertion, deletion, amino acid substitution or other targeted mutation. The location of the disruption in the protein may be, for example, within the N-terminal portion of the protein or within the C-terminal portion of the protein. The disrupted Pex protein will have impaired activity with respect to the Pex protein that was not disrupted, and can be non-functional. A disruption in a native gene encoding a Pex protein also includes alternate means that result in low or lack of expression of the Pex protein, such as could result via manipulating the regulatory sequences, transcription and translation factors and/or signal transduction pathways or by use of sense, antisense or RNAi technology, etc.

As used herein, the term "Pex-disrupted organism" refers to any oleaginous eukaryotic organism comprising genes that encode a functional polyunsaturated fatty acid biosynthetic pathway and having a disruption, as defined above, in a native gene that encodes a peroxisome biogenesis factor protein,

The term "lipids" refer to any fat-soluble (i.e., lipophilic), naturally-occurring molecule. Lipids are a diverse group of compounds that have many key biological functions, such as structural components of cell membranes, energy storage sources and intermediates in signaling pathways. Lipids may be broadly defined as hydrophobic or amphiphilic small molecules that originate entirely or in part from either ketoacyl or isoprene groups. A general overview of lipids, based on the Lipid Metabolites and Pathways Strategy (LIPID MAPS) classification system (National Institute of General Medical Sciences, Bethesda, MD), is shown below in Table 2.

Table 2

Overview Of Lipid Classes

Structural Building Block	Lipid Category	Examples Of Lipid Classes
Derived from condensation of ketoacyl subunits	Fatty Acyls	Includes fatty acids, eicosanoids, fatty esters and fatty amides
	Glycerolipids	Includes mainly of mono-, di- and tri-substituted glycerols, the most well-known being the fatty acid esters of glycerol ["triacylglycerols"]
	Glycero-phospholipids or Phospholipids	Includes phosphatidylcholine, phosphatidylethanolamine, phosphatidylserine, phosphatidylinositols and phosphatidic acids
	Sphingolipids	Includes ceramides, phosphosphingolipids (e.g., sphingomyelins), glycosphingolipids (e.g., gangliosides), sphingosine, cerebroside
	Saccharolipids	Includes acylaminosugars, acylaminosugar glycans, acyltrehaloses, acyltrehalose glycans
	Polyketides	Includes halogenated acetogenins, polyenes, linear tetracyclines, polyether antibiotics, flavonoids, aromatic polyketides
Derived from condensation of isoprene subunits	Sterol Lipids	Includes sterols (e.g., cholesterol), C18 steroids (e.g., estrogens), C19 steroids (e.g., androgens), C21 steroids (e.g., progestogens, glucocorticoids and mineralocorticoids), secosteroids, bile acids
	Prenol Lipids	Includes isoprenoids, carotenoids, quinones, hydroquinones, polyprenols, hopanoids

The term "total lipid fraction" of cells herein refers to all esterified fatty acids of the cell. Various subfractions within the total lipid fraction
5 can be isolated, including the triacylglycerol ["oil"] fraction, phosphatidylcholine fraction and the phosphatidylethanolamine fraction, although this is by no means inclusive of all sub-fractions.

"Lipid bodies" refer to lipid droplets that are bound by a monolayer of phospholipid and, usually, by specific proteins. These organelles are
10 sites where most organisms transport/store neutral lipids. Lipid bodies are thought to arise from microdomains of the endoplasmic reticulum that

contain TAG biosynthesis enzymes. Their synthesis and size appear to be controlled by specific protein components.

“Neutral lipids” refer to those lipids commonly found in cells in lipid bodies as storage fats and oils and are so called because at cellular pH, the lipids bear no charged groups. Generally, they are completely non-polar with no affinity for water. Neutral lipids generally refer to mono-, di-, and/or triesters of glycerol with fatty acids, also called monoacylglycerol, diacylglycerol or triacylglycerol, respectively, or collectively, acylglycerols. A hydrolysis reaction must occur to release free fatty acids from acylglycerols.

The terms “triacylglycerols” [“TAGs”] and “oil” are interchangeable and refer to neutral lipids composed of three fatty acyl residues esterified to a glycerol molecule. TAGs can contain long chain PUFAs, as well as shorter saturated and unsaturated fatty acids and longer chain saturated fatty acids. The TAG fraction of cells is also referred to as the “oil fraction”, and “oil biosynthesis” generically refers to the synthesis of TAGs in the cell. The oil or TAG fraction is a sub-fraction of the total lipid fraction, although also it constitutes a major part of the total lipid content, measured as the weight of total fatty acids in the cell as a percent of the dry cell weight [see below], in oleaginous organisms. The fatty acid composition in the oil [“TAG”] fraction and the fatty acid composition of the total lipid fraction are generally similar. Thus, an increase or decrease in the concentration of PUFAs in the total lipid fraction will correspond with an increase or decrease in the concentration of PUFAs in the oil [“TAG”] fraction, and vice versa.

The term “total fatty acids” [“TFAs”] herein refer to the sum of all cellular fatty acids that can be derivitized to fatty acid methyl esters [“FAMES”] by the base transesterification method (as known in the art) in a given sample, which may be the total lipid fraction or the oil fraction, for example. Thus, total fatty acids include fatty acids from neutral and polar lipid fractions, including the phosphatidylcholine fraction, the phosphatidyletanolamine fraction and the diacylglycerol, monoacylglycerol and triacylglycerol [“TAG or oil”] fractions but not free fatty acids.

3The term “total lipid content” of cells is a measure of TFAs as a percent of the dry cell weight [“DCW”]. Thus, total lipid content [“TFAs % DCW”] is equivalent to, e.g., milligrams of total fatty acids per 100 milligrams of DCW.

5 Generally, the concentration of a fatty acid is expressed herein as a weight percent of TFAs [“% TFAs”], e.g., milligrams of the given fatty acid per 100 milligrams of TFAs. Unless otherwise specifically stated in the disclosure herein, reference to the percent of a given fatty acid with respect to total lipids is equivalent to concentration of the fatty acid as %
10 TFAs (e.g., % EPA of total lipids is equivalent to EPA % TFAs).

In some cases, it is useful to express the content of a given fatty acid(s) in a cell as its percent of the dry cell weight [“% DCW”]. Thus, for example, eicosapentaenoic acid % DCW would be determined according to the following formula: (eicosapentaenoic acid % TFAs) * (TFA %
15 DCW)]/100.

The terms “lipid profile” and “lipid composition” are interchangeable and refer to the amount of an individual fatty acid contained in a particular lipid fraction, such as in the total lipid fraction or the oil [“TAG”] fraction, wherein the amount is expressed as a percent of TFAs. The sum of each
20 individual fatty acid present in the mixture should be 100.

As used herein, the term “fold increase” refers to an increase obtained by multiplying by a number. For example, multiplying by 1.3 a quantity, an amount, a concentration, a weight percent, etc. provides a 1.3 fold increase.

25 The term “fatty acids” refers to long chain aliphatic acids (alkanoic acids) of varying chain lengths, from about C₁₂ to C₂₂, although both longer and shorter chain-length acids are known. The predominant chain lengths are between C₁₆ and C₂₂. The structure of a fatty acid is represented by a simple notation system of “X:Y”, where X is the total
30 number of carbon [“C”] atoms in the particular fatty acid and Y is the number of double bonds. Additional details concerning the differentiation between “saturated fatty acids” versus “unsaturated fatty acids”, “monounsaturated fatty acids” versus “polyunsaturated fatty acids”

["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. Pat. No. 7,238,482.

Nomenclature used to describe PUFAs herein is given in Table 3. 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 are used throughout the specification and the chemical name of each compound.

Table 3

Nomenclature of Polyunsaturated Fatty Acids And Precursors

Common Name	Abbreviation	Chemical Name	Shorthand Notation
Myristic	--	Tetradecanoic	14:0
Palmitic	Palmitate	Hexadecanoic	16:0
Palmitoleic	--	9-hexadecenoic	16:1
Stearic	--	Octadecanoic	18:0
Oleic	--	<i>cis</i> -9-octadecenoic	18:1
Linoleic	LA	<i>cis</i> -9, 12-octadecadienoic	18:2 ω -6
γ -Linolenic	GLA	<i>cis</i> -6, 9, 12-octadecatrienoic	18:3 ω -6
Eicosadienoic	EDA	<i>cis</i> -11, 14-eicosadienoic	20:2 ω -6
Dihomo- γ -Linolenic	DGLA	<i>cis</i> -8, 11, 14-eicosatrienoic	20:3 ω -6
Arachidonic	ARA	<i>cis</i> -5, 8, 11, 14-eicosatetraenoic	20:4 ω -6
α -Linolenic	ALA	<i>cis</i> -9, 12, 15-octadecatrienoic	18:3 ω -3
Stearidonic	STA	<i>cis</i> -6, 9, 12, 15-octadecatetraenoic	18:4 ω -3
Eicosatrienoic	ETrA	<i>cis</i> -11, 14, 17-eicosatrienoic	20:3 ω -3
Sciadonic	SCI	<i>cis</i> -5, 11, 14-eicosatrienoic	20:3b ω -6
Juniperonic	JUP	<i>cis</i> -5, 11, 14, 17-eicosatetraenoic	20:4b ω -3
Eicosa-tetraenoic	ETA	<i>cis</i> -8, 11, 14, 17-eicosatetraenoic	20:4 ω -3
Eicosa-pentaenoic	EPA	<i>cis</i> -5, 8, 11, 14, 17-eicosapentaenoic	20:5 ω -3
Docosatrienoic	DRA	<i>cis</i> -10,13,16-	22:3 ω -3

		docosatrienoic	
Docosa- tetraenoic	DTA	<i>cis</i> -7,10,13,16- docosatetraenoic	22:4 ω -3
Docosa- pentaenoic	DPA ω -6	<i>cis</i> -4,7,10,13,16- docosapentaenoic	22:5 ω -6
Docosa- pentaenoic	DPA	<i>cis</i> -7, 10, 13, 16, 19- docosapentaenoic	22:5 ω -3
Docosa- hexaenoic	DHA	<i>cis</i> -4, 7, 10, 13, 16, 19- docosahexaenoic	22:6 ω -3

Although the ω -3/ ω -6 PUFAs listed in Table 3 are the most likely to be accumulated in the oil fractions of oleaginous yeast using the methods described herein, this list should not be construed as limiting or as complete.

- 5 As used herein, the terms "a combination of polyunsaturated fatty acids" or "any combination of polyunsaturated fatty acids" refers to a mixture of any two or more of the polyunsaturated fatty acids listed above in Table 3. Such combination has the attributes of a concentration and of a weight percent that can be measured relative to a variety of
- 10 concentrations or weight percents in the cell, including relative to the weight percent of the total fatty acids in the cell.

A metabolic pathway, or biosynthetic pathway, in a biochemical sense, can be regarded as a series of chemical reactions occurring in order within a cell, catalyzed by enzymes, to achieve either the formation

15 of a metabolic product to be used or stored by the cell, or the initiation of another metabolic pathway, which is termed "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.

20 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 DPA ω -6 and ω -3 fatty acids such as ALA, STA, ETrA, ETA, EPA, DPA and DHA. This process is well described in the literature. See e.g., Int'. App. Pub.No. WO 2006/052870. Briefly, this

25 process involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the elongated molecule through the addition of double bonds, via a series of special elongation and

desaturation enzymes termed “PUFA biosynthetic pathway enzymes” that are present in the endoplasmic reticulum membrane. More specifically, “PUFA biosynthetic pathway enzymes” refer to any of the following enzymes (and genes which encode them) associated with the

- 5 biosynthesis of a PUFA, including: a $\Delta 4$ desaturase, a $\Delta 5$ desaturase, a $\Delta 6$ desaturase, a $\Delta 12$ desaturase, a $\Delta 15$ desaturase, a $\Delta 17$ desaturase, a $\Delta 9$ desaturase, a $\Delta 8$ desaturase, a $\Delta 9$ elongase, a $C_{14/16}$ elongase, a $C_{16/18}$ elongase, a $C_{18/20}$ elongase and/or a $C_{20/22}$ elongase.

The term “ ω -3/ ω -6 fatty acid biosynthetic pathway” refers to a set of
10 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
15 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, such that one portion generates only ω -3 fatty acids and the other portion, only ω -6 fatty acids. That portion that generates only ω -3 fatty acids is referred to herein as the
20 ω -3 fatty acid biosynthetic pathway, whereas that portion that generates only ω -6 fatty acids is referred to herein as the ω -6 fatty acid biosynthetic pathway.

The term “functional” as used herein relating to the ω -3/ ω -6 fatty acid biosynthetic pathway, means that some (or all) of the genes in the
25 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 of the genes listed in the above paragraph are required, as a number of fatty acid products require only the expression of a subset of
30 the genes of this pathway.

The term “ $\Delta 6$ desaturase/ $\Delta 6$ elongase pathway” refers to a PUFA biosynthetic pathway that minimally includes at least one $\Delta 6$ desaturase

and at least one C_{18/20} 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.

5 The term “ $\Delta 9$ elongase/ $\Delta 8$ desaturase pathway” refers to a PUFA biosynthetic pathway that minimally includes at least one $\Delta 9$ elongase and at least one $\Delta 8$ 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
10 elongases, ARA, EPA, DPA and DHA may also be synthesized.

 The term “desaturase” refers to a polypeptide that can desaturate adjoining carbons in a fatty acid by removing a hydrogen from one of the adjoining carbons and thereby introducing a double bond between them. Desaturation produces a fatty acid or precursor of interest. Despite use of
15 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. Of particular interest herein are: 1) $\Delta 5$ desaturases that catalyze the conversion of the substrate fatty acid, DGLA, to ARA and/or
20 of the substrate fatty acid, ETA, to EPA; 2) $\Delta 17$ desaturases that desaturate a fatty acid between the 17th and 18th carbon atom numbered from the carboxyl-terminal end of the molecule and which, for example, catalyze the conversion of the substrate fatty acid, ARA, to EPA and/or the conversion of the substrate fatty acid, DGLA, to ETA; 3) $\Delta 6$ desaturases
25 that catalyze the conversion of the substrate fatty acid, LA, to GLA and/or the conversion of the substrate fatty acid, ALA, to STA; 4) $\Delta 12$ desaturases that catalyze the conversion of the substrate fatty acid, oleic acid, to LA; 5) $\Delta 15$ desaturases that catalyze the conversion of the substrate fatty acid, LA, to ALA and/or the conversion of the substrate
30 fatty acid, GLA, to STA; 6) $\Delta 4$ desaturases that catalyze the conversion of the substrate fatty acid, DPA, to DHA and/or the conversion of the substrate fatty acid, DTA, to DPAn-6; 7) $\Delta 8$ desaturases that catalyze the

conversion of the substrate fatty acid, EDA, to DGLA and/or the conversion of the substrate fatty acid, ETrA, to ETA; and, 8) $\Delta 9$ desaturases that catalyze the conversion of the substrate fatty acid, palmitate, to palmitoleic acid (16:1) and/or the conversion of the substrate fatty acid, stearic acid, to oleic acid. $\Delta 15$ and $\Delta 17$ 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). 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.

The term “elongase” refers to a polypeptide that can elongate a fatty acid carbon chain to produce an acid 2 carbons longer than the fatty acid substrate that the elongase acts upon. This process of elongation occurs in a multi-step mechanism in association with fatty acid synthase, as described in U.S. Pat. App. Pub. No. 2005/0132442 and Int'l App. Pub. No. WO 2005/047480. Examples of reactions catalyzed by elongase systems are the conversion of GLA to DGLA, STA to ETA and EPA to DPA. In general, the substrate selectivity of elongases is somewhat broad but segregated by both chain length and the degree and type of unsaturation. For example, a $C_{14/16}$ elongase utilizes a C_{14} substrate e.g., myristic acid, a $C_{16/18}$ elongase utilizes a C_{16} substrate e.g., palmitate, a $C_{18/20}$ elongase [also known as a $\Delta 6$ elongase as the terms can be used interchangeably] utilizes a C_{18} substrate e.g., GLA or STA, and a $C_{20/22}$ elongase utilizes a C_{20} substrate e.g., EPA. In like manner, a $\Delta 9$ elongase is able to catalyze the conversion of LA and ALA to EDA and ETrA, respectively. It is important to note that some elongases have broad specificity and thus a single enzyme may be capable of catalyzing several elongase reactions. For example a single enzyme may thus act as both a $C_{16/18}$ elongase and a $C_{18/20}$ elongase.

The terms “conversion efficiency” and “percent substrate conversion” refer to the efficiency by which a particular enzyme, such as a desaturase, can convert substrate to product. The conversion efficiency is measured according to the following formula: ([product]/[substrate +
5 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 oil (Weete, In: Fungal Lipid Biochemistry, 2nd Ed., Plenum, 1980).

10 The term “oleaginous yeast” refers to those microorganisms classified as yeasts that can make oil, that is, TAGs. Generally, the cellular oil or TAG content of oleaginous microorganisms 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
15 gradually decreases during the late stationary and death phases (Yongmanitchai and Ward, *Appl. Environ. Microbiol.*, 57:419-25 (1991)). Oleaginous microorganisms as referred to herein typically accumulate in excess of about 25% of their dry cell weight as oil or TAGs. Examples of oleaginous yeast include, but are not limited to, the following genera:
20 *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

As used herein, the terms “isolated nucleic acid fragment” and “isolated nucleic acid molecule” are used interchangeably and refer to a polymer of RNA or DNA that is single- or double-stranded, optionally
25 containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid fragment in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

A nucleic acid fragment is “hybridizable” to another nucleic acid fragment, such as a cDNA, genomic DNA, or RNA molecule, when a
30 single-stranded form of the nucleic acid fragment can anneal to the other nucleic acid fragment under the appropriate conditions of temperature and solution ionic strength. Hybridization and washing conditions are well known and exemplified in Sambrook, J., Fritsch, E. F. and Maniatis, T.

Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989).

A “substantial portion” of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a
5 polypeptide or the nucleotide sequence of a gene to putatively identify that polypeptide or gene, either by manual evaluation of the sequence by one skilled in the art, or by computer-automated sequence comparison and identification using algorithms such as BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., *J. Mol. Biol.*, 215:403-410 (1993)). In
10 general, a sequence of ten or more contiguous amino acids or of thirty or more contiguous nucleotides is necessary in order to putatively identify a polypeptide or nucleic acid sequence as homologous to a known protein or gene. Moreover, with respect to nucleotide sequences, gene specific oligonucleotide probes comprising 20-30 contiguous nucleotides may be
15 used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation, such as *in situ* hybridization of microbial colonies or bacteriophage plaques. In addition, short oligonucleotides of 12-15 bases may be used as amplification primers in PCR in order to obtain a particular nucleic acid fragment comprising the
20 primers. Accordingly, a “substantial portion” of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid fragment comprising the sequence.

The term “complementary” is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another.
25 For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine.

The terms “homology” and “homologous” 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
30 to mediate gene expression or produce a certain phenotype. These terms also refer to modifications of the Pex nucleic acid fragments described herein, 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.

Moreover, the skilled artisan recognizes that homologous nucleic acid sequences are also defined by their ability to hybridize, under
5 moderately stringent conditions, such as 0.5 X 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 thereto.

“Codon degeneracy” refers to the nature in the genetic code permitting variation of the nucleotide sequence without effecting the amino
10 acid sequence of an encoded polypeptide. 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
15 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 oligonucleotide building blocks are annealed and then ligated to form gene segments that are then enzymatically assembled
20 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
25 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 which may refer to the coding region alone or may include regulatory sequences preceding (5' non-coding sequences) and following
30 (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
5 gene” refers to a native gene in its natural location in the genome of an organism. A “foreign” gene refers to a gene that is introduced into the host organism by gene transfer. Foreign genes can comprise native genes inserted into a non-native organism, native genes introduced into a new location within the native host, or chimeric genes. A “transgene” is a gene
10 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. “Suitable regulatory sequences” refer to
15 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 promoters, enhancers, silencers, 5' untranslated leader
20 sequence (e.g., between the transcription start site and the translation initiation codon), 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
25 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
30 tissues or cell types, or at different stages of development, or in response to different environmental or physiological conditions. Promoters that cause a gene to be expressed in most cell types at most times are commonly referred to as “constitutive promoters”. It is further recognized

that since in most cases the exact boundaries of regulatory sequences have not been completely defined, DNA fragments of different lengths may have identical promoter activity.

The terms “3' non-coding sequences” and “transcription terminator” 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 or it may be a RNA sequence derived from post-transcriptional processing of the primary transcript and is referred to as the mature RNA. “Messenger RNA” or “mRNA” refers to the RNA that is without introns and which can be translated into protein by the cell. “cDNA” refers to a double-stranded DNA that is complementary to, and derived from, mRNA. “Sense” RNA refers to RNA transcript that includes the mRNA and so can be translated into protein by the cell. “Antisense RNA” refers to a 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. Pat. No. 5,107,065; Int'l. App. Pub. No. WO 99/28508). 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, or the coding sequence. “Functional RNA” refers to antisense RNA, ribozyme RNA, or other RNA that is not translated and yet has an effect on cellular processes.

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. That is, the coding sequence is under the transcriptional control of the promoter. Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

The term “expression”, as used herein, refers to the transcription
5 and stable accumulation of sense (mRNA) or antisense RNA derived from nucleic acid fragments. Expression may also refer to translation of mRNA into a polypeptide.

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

“Transformation” refers to the transfer of a nucleic acid molecule
15 into a host organism, resulting in genetically stable inheritance. The nucleic acid molecule may be a plasmid that replicates autonomously, for example, or, it may integrate into the genome of the host organism. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” or “recombinant” or “transformed” organisms.

20 “Stable transformation” refers to the transfer of a nucleic acid fragment into a genome of a host organism, including both nuclear and organellar genomes, resulting in genetically stable inheritance. In contrast, “transient transformation” refers to the transfer of a nucleic acid fragment into the nucleus, or DNA-containing organelle, of a host
25 organism resulting in gene expression without integration or stable inheritance. Host organisms containing the transformed nucleic acid fragments are referred to as “transgenic” organisms.

The terms “plasmid” and “vector” refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of
30 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 that 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., open reading frame ["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.

The term “percent identity” refers to a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. “Identity” also means the degree of sequence relatedness between polypeptide or polynucleotide sequences, as the case may be, as determined by the percentage of match between compared sequences. “Percent identity” and “percent similarity” can be readily calculated by known methods, including but not limited to those described in: 1) Computational Molecular Biology (Lesk, A. M., Ed.) Oxford University: NY (1988); 2) Biocomputing: Informatics and Genome Projects (Smith, D. W., Ed.) Academic: NY (1993); 3) Computer Analysis of Sequence Data, Part I (Griffin, A. M., and Griffin, H. G., Eds.) Humana: NJ (1994); 4) Sequence Analysis in Molecular Biology (von Heinje, G., Ed.) Academic (1987); and, 5) Sequence Analysis Primer (Gribskov, M. and Devereux, J., Eds.) Stockton: NY (1991).

Preferred methods to determine percent identity are designed to give the best match between the sequences tested. Methods to determine percent identity and percent similarity are codified in publicly available computer programs. Sequence alignments and percent identity

calculations may be performed using the MegAlign™ program of the LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences is performed using the “Clustal method of alignment” which encompasses several varieties of the
5 algorithm including the “Clustal V method of alignment” and the “Clustal W method of alignment” (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™ v6.1 program of the LASERGENE bioinformatics computing suite (DNASTAR Inc.). After alignment of the
10 sequences using either Clustal program, it is possible to obtain a “percent identity” by viewing the “sequence distances” table in the program.

It is well understood by one skilled in the art that various measures of sequence percent identity are useful in identifying polypeptides, from other species, wherein such polypeptides have the same or similar
15 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 suitable nucleic acid fragments (isolated polynucleotides) encoding polypeptides in
20 methods and host cells described herein, 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%. In some cases, suitable nucleic acid
25 fragments (isolated polynucleotides) encode polypeptides that are at least about 70% identical, preferably at least about 75% identical, and more preferably at least about 80% identical to the amino acid sequences reported herein. Preferred nucleic acid fragments encode amino acid sequences that are at least about 85% identical to the amino acid
30 sequences reported herein. More preferred nucleic acid fragments encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid

fragments that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein.

Suitable nucleic acid fragments not only have the above homologies but typically encode a polypeptide having at least 50 amino acids, preferably at least 100 amino acids, more preferably at least 150 amino acids, still more preferably at least 200 amino acids, and most preferably at least 250 amino acids.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software include, but is not limited to: 1) the GCG suite of programs (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI); 2) BLASTP, BLASTN, BLASTX (Altschul et al., *J. Mol. Biol.*, 215:403-410 (1990)); 3) DNASTAR (DNASTAR, Inc. Madison, WI); 4) Sequencher (Gene Codes Corporation, Ann Arbor, MI); and, 5) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Editor(s): Suhai, Sandor. Plenum: New York, NY). Within this description, whenever sequence analysis software is used for analysis, the analytical results are based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" means any set of values or parameters that originally load with the software when first initialized.

Standard recombinant DNA and molecular cloning techniques used herein are well known in the art and are described by Sambrook, J., Fritsch, E. F. and Maniatis, T., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1989) (hereinafter "Maniatis"); by Silhavy, T. J., Bennis, 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).

An Overview: 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
5 palmitate (16:0) in oleaginous microorganisms, is described in detail in U.S. Pat. No. 7,238,482. Palmitate is the precursor of longer-chain saturated and unsaturated fatty acid derivatives, which are formed through the action of elongases and desaturases (FIG. 1).

TAGs, the primary storage unit for fatty acids, are formed by a
10 series of reactions that involve: 1) esterification of one molecule of acyl-CoA to glycerol-3-phosphate via an acyltransferase to produce lysophosphatidic acid; 2) 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
15 acid phosphatase to yield 1,2-diacylglycerol ["DAG"]; and, 4) addition of a third fatty acid by the action of an acyltransferase to form the 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. Some non-limiting examples of fatty acids that can be
20 incorporated into TAGs by acyltransferases include: capric (10:0), lauric (12:0), myristic (14:0), palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), vaccenic (18:1), LA (18:2), eleostearic (18:3), GLA (18:3), ALA (18:3), STA (18:4), arachidic (20:0), EDA (20:2), DGLA (20:3), ETrA (20:3), ARA (20:4), ETA (20:4), EPA (20:5), behenic (22:0), DPA (22:5),
25 DHA (22:6), lignoceric (24:0), nervonic (24:1), cerotic (26:0) and montanic (28:0) fatty acids. In the methods and host cells described herein, incorporation of "long-chain" PUFAs into TAGs may be most desirable, wherein long-chain PUFAs include any fatty acid derived from an 18:1 substrate having at least 18 carbons in length, i.e., C₁₈ or greater. This
30 also includes hydroxylated fatty acids, epoxy fatty acids and conjugated linoleic acid.

Although most PUFAs are incorporated into TAGs as neutral lipids and are stored in lipid bodies, it is important to note that a measurement of

the total PUFAs within an oleaginous organism should include those PUFAs that are located in the phosphatidylcholine fraction, phosphatidylethanolamine fraction, and triacylglycerol, also known as the TAG or oil, fraction.

5 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
10 elongation enzymes present in the endoplasmic reticulum 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, FIG. 1 depicts the pathways described below. All pathways require the initial conversion of oleic acid to linoleic acid ["LA"],
15 the first of the ω -6 fatty acids, by a Δ 12 desaturase. Then, using the " Δ 6 desaturase/ Δ 6 elongase pathway" and LA as substrate, long-chain ω -6 fatty acids are formed as follows: 1) LA is converted to γ -linolenic acid ["GLA"] by a Δ 6 desaturase; 2) GLA is converted to dihomo- γ -linolenic acid ["DGLA"] by a $C_{18/20}$ elongase; 3) DGLA is converted to arachidonic acid
20 ["ARA"] by a Δ 5 desaturase; 4) ARA is converted to docosatetraenoic acid ["DTA"] by a $C_{20/22}$ elongase; and, 5) DTA is converted to docosapentaenoic acid ["DPA ω -6"] by a Δ 4 desaturase.

Alternatively, the " Δ 6 desaturase/ Δ 6 elongase pathway" can use α -linolenic acid ["ALA"] as substrate to produce long-chain ω -3 fatty acids as
25 follows: 1) LA is converted to ALA, the first of the ω -3 fatty acids, by a Δ 15 desaturase; 2) ALA is converted to stearidonic acid ["STA"] by a Δ 6 desaturase; 3) STA is converted to eicosatetraenoic acid ["ETA"] by a $C_{18/20}$ elongase; 4) ETA is converted to eicosapentaenoic acid ["EPA"] by a Δ 5 desaturase; 5) EPA is converted to docosapentaenoic acid ["DPA"]
30 by a $C_{20/22}$ elongase; and, 6) DPA is converted to docosahexaenoic acid ["DHA"] by a Δ 4 desaturase. Optionally, ω -6 fatty acids may be converted

to ω -3 fatty acids. For example, ETA and EPA are produced from DGLA and ARA, respectively, by Δ 17 desaturase activity.

Alternate pathways for the biosynthesis of ω -3/ ω -6 fatty acids utilize Δ 9 elongase and Δ 8 desaturase, that is, the “ Δ 9 elongase/ Δ 8 desaturase pathway”. More specifically, LA and ALA may be converted to EDA and ETrA, respectively, by a Δ 9 elongase. A Δ 8 desaturase then converts EDA to DGLA and/or ETrA to ETA. Downstream PUFAs are subsequently formed as described above.

The host organism herein must possess the ability to produce PUFAs, either naturally or via techniques of genetic engineering. Although many microorganisms can synthesize PUFAs (including ω -3/ ω -6 fatty acids) in the ordinary course of cellular metabolism, some of whom could be commercially cultured, few to none of these organisms produce oils having a desired oil content and composition for use in pharmaceuticals, dietary substitutes, medical foods, nutritional supplements, other food products, industrial oleochemicals or other end-use applications. Thus, there is increasing emphasis on the ability to engineer microorganisms for production of “designer” lipids and oils, wherein the fatty acid content and composition are carefully specified by genetic engineering. On this basis, it is expected that the host likely comprises heterologous genes encoding a functional PUFA biosynthetic pathway but not necessarily.

If the host organism does not natively produce the desired PUFAs or possess the desired lipid profile, one skilled in the art is familiar with the considerations and techniques necessary to introduce one or more expression cassettes encoding appropriate enzymes for PUFA biosynthesis into the host organism of choice. Numerous teachings are provided in the literature to one of skill for so introducing such expression cassettes into various host organisms. Some references using the host organism *Yarrowia lipolytica* are provided as follows: U.S. Pat. No. 7,238,482, Int'l. App. Pub. No. WO 2006/033723, Pat. Appl. Pub. No. US-2006-0094092, Pat. Appl. Pub. No. US-2006-0115881-A1 and Pat. Appl. Pub. No. US-2006-0110806-A1. This list is not exhaustive and should not be construed as limiting.

Briefly, a variety of ω -3/ ω -6 PUFA products can be produced prior to their transfer to TAGs, depending on the fatty acid substrate and the particular genes of the ω -3/ ω -6 fatty acid biosynthetic pathway that are present in or transformed into the host cell. As such, production of the
5 desired fatty acid product can occur directly or indirectly. Direct production occurs when the fatty acid substrate is converted directly into the desired fatty acid product without any intermediate steps or pathway intermediates. Indirect production occurs when multiple genes encoding the PUFA biosynthetic pathway may be used in combination such that a
10 series of reactions occur to produce a desired PUFA. Specifically, it may be desirable to transform an oleaginous yeast with an expression cassette comprising a Δ 12 desaturase, Δ 6 desaturase, a $C_{18/20}$ elongase, a Δ 5 desaturase and a Δ 17 desaturase for the overproduction of EPA. See U.S. Pat. No. 7,238,482 and Int'l. App. Pub. No. WO 2006/052870. As is
15 well known to one skilled in the art, various other combinations of genes encoding enzymes of the PUFA biosynthetic pathway may be useful to express in an oleaginous organism (see FIG. 1). The particular genes included within a particular expression cassette depend on the host organism, its PUFA profile and/or desaturase/elongase profile, the
20 availability of substrate and the desired end product(s).

A number of candidate genes having the desired desaturase and/or elongase activities can be identified according to publicly available literature, such as GenBank, the patent literature, and experimental analysis of organisms having the ability to produce PUFAs. Useful
25 desaturase and elongase sequences may be derived from any source, e.g., isolated from a natural source such as from bacteria, algae, fungi, oomycete, yeast, plants, animals, etc., produced via a semi-synthetic route or synthesized *de novo*. Following the identification of these candidate genes, considerations for choosing a specific polypeptide having
30 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 is modified after its production, such as 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. Pat. No. 7,238,482. It may 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 un-purified oils produced in a host cell is typically a mixture of various PUFAs consisting of the desired ω -3/ ω -6 fatty acid, as well as various upstream intermediary PUFAs. Thus, the conversion efficiency of each enzyme is also a variable to consider when optimizing biosynthesis of a desired fatty acid.

Peroxisome Biogenesis And Pex Genes

As previously described, peroxisomes are ubiquitous organelles found in all eukaryotic cells. Their primary role is the degradation of various substances within a localized organelle of the cell, such as toxic compounds, fatty acids, etc. For example, the process of β -oxidation, wherein fatty acid molecules are broken down to ultimately produce free molecules of acetyl-CoA (which are exported back to the cytosol), can occur in peroxisomes. Although the process of β -oxidation in mitochondria results in ATP synthesis, β -oxidation in peroxisomes causes the transfer of high-potential electrons to O_2 and results in the formation of H_2O_2 , which is subsequently converted to water and O_2 by peroxisome catalases. Very long chain, such as C_{18} to C_{22} , fatty acids undergo initial β -oxidation in peroxisomes, followed by mitochondrial β -oxidation.

The proteins responsible for importing proteins by means of ATP hydrolysis through the peroxisomal membrane are known as peroxisome biogenesis factor proteins, or "peroxins". These peroxisome biogenesis factor proteins also include those proteins involved in peroxisome biogenesis/assembly. The gene acronym for peroxisome biogenesis factor proteins is Pex; and, a system for nomenclature is described by Distel et al., *J. Cell Biol.*, 135:1-3 (1996). At least 32 different Pex genes have been identified so far in various eukaryotic organisms. In fungi,

however, the recent review of Kiel et al. (*Traffic*, 7:1291-1303 (2006)) suggests that the minimal requirement for peroxisome biogenesis/matrix protein import is numbered as 17, thereby requiring only Pex1p, Pex2p, Pex3p, Pex4p, Pex5p, Pex6p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, 5 Pex14p, Pex17p, Pex19p, Pex20p, Pex22p and Pex26p. These proteins act in a coordinated fashion to proliferate (duplicate) peroxisomes and import proteins via translocation into peroxisomes (reviewed in Waterham, H.R. and J.M. Cregg. *BioEssays*. 19(1):57-66 (1996)).

Many Pex genes were initially isolated from the analysis of mutants 10 that demonstrated abnormal peroxisomal functions or structures. With the availability of complete genome sequences, however, it is becoming increasingly easy to identify Pex genes via computer sequence searches based on homology. Kiel et al. (*Traffic*, 7:1291-1303 (2006)) cite strong conservation of the peroxisome biogenesis machinery, despite occasional 15 low sequence similarity. More specifically, within the yeast and filamentous fungi, their data indicate that almost all Pex proteins identified thus far are conserved. Table 4, below, shows peroxisome biogenesis factor proteins identified by Kiel et al. (*supra*) in *Saccharomyces cerevisiae*, *Candida glabrata*, *Ashbya gossypii*, *Kluyveromyces lactis*, 20 *Candida albicans*, *Debaryomyces hansenii*, *Pichia pastoris*, *Hansenula polymorpha*, *Yarrowia lipolytica*, *Aspergillus fumigatus*, *Aspergillus nidulans*, *Penicillium chrysogenum*, *Magnaporthe grisea*, *Neurospora crassa*, *Gibberella zeae*, *Ustilago maydis*, *Cryptococcus neoformans* var. *neoformans* and *Schizosaccharomyces pombe*.

Table 4
GenBank Accession Numbers Of Fungal Peroxisome Biogenesis Factor Proteins
[Recreated From Table 2 of Kiel et al., (*Traffic*, 7:1291-1303 (2006))]

	<i>Saccharo- myces cerevisiae</i>	<i>Candida glabrata</i>	<i>Ashbya gossypii</i>	<i>Kluyvero- myces lactis</i>	<i>Candida albicans</i>	<i>Debaryo- myces hansenii</i>	<i>Pichia pastoris</i>	<i>Hansenula polymorpha</i>	<i>Yarrowia lipolytica</i>
Pex1p	CAA82041	CAG60131	AAS53742	CAH02218	EAL02496	CAG89689	CAA85450	AAD52811	CAG82178
Pex2p	CAA89508	CAG60461	AAS50677	CAH00186	EAK95929	CAG85956	CAA65646	AAT97412	CAG77647
Pex3p	AAB64764	CAG62379	AAS52217	CAG99801	EAK94771	CAG89890	CAA96530	AAC49471	CAG78565
Pex3Bp	—	—	—	—	—	—	na	—	CAG83356
Pex4p	CAA97146	CAG60639	AAS53685	CAG99212	EAL03336	CAG87262	AAA53634	AAC16238	CAG79130
Pex5p	CAA89730	CAG61665	AAS53824	CAH01742	EAK94251	CAG89098	AAB40613	AAC49040	CAG78803
Pex5Bp	—	CAG61076	—	—	—	—	na	—	—
Pex5Cp	CAA89120 (Ymr018wp)	—	—	—	—	—	na	—	—
Pex5/20p	—	—	—	—	—	—	na	—	—
Pex5Rp	—	—	—	—	—	—	na	—	—
Pex6p	AAA16574	CAG58438	AAS54884	CAG99125	EAK95956	CAG87108	CAA80278	AAD52812	CAG82306
Pex7p	CAA57183	CAG57936	AAS54301	CAG99215	EAK95226	CAG87150	AAC08303	ABA64462	CAG78389
Pex8p	CAA97079	CAG61238	AAS52889	CAH01253	EAK91777, EAK91778*	CAG89446	AAC41653	CAA82928	CAG80447
Pex9p	ORF wrongly identified	—	—	—	—	—	—	—	—
Pex10p	AAB64453	CAG62699	AAS53069	CAG99788	Translation of AACQ- 01000128, nucleotides 37281-36306	CAG89101	AAB09086	CAA86101	CAG81606

								(contains intron)						
Pex12p	CAA89129	CAG62649	AAS50837	CAG99378	EAL00707	CAG84342	AAC49402	AAM66157	CAG81532					
Pex13p	AAB46885	CAG57840	AAS51456	CAG99931	EAK97421	CAG86337	AAB09087	DQ345349	CAG81789					
Pex14p	AAS56829	CAG58828	AAS54871	CAG99440	EAK90926	CAG91028	AAG28574	AAB40596	CAG79323					
Pex15p	CAA99046	CAG58938	AAS51506	CAG98135	—	—	na	—	—					
Pex16p	—	—	—	—	—	—	na	—	CAG79622					
Pex17p	CAA96116	CAG61398	AAS50595	CAH01010	EAK95385	CAG86168	AAF19606	DQ345350	CAG84025					
Pex14/17p	—	—	—	—	—	—	na	—	—					
Pex18p	AAB68992	—	—	—	—	—	na	—	—					
Pex19p	CAA98630	CAG58359	AAS52741	CAG99258	EAK97275	CAG84799	AAD43507	AAK84070	AAK84827					
Pex20p	—	—	—	—	EAK91603, EAK94766*	CAG87898	AAX11696	AAX14715	CAG79226					
Pex21p	CAA97267	CAG59241	AAS51769	CAG99735	—	—	na	—	—					
Pex21Bp	—	CAG60281	—	—	—	—	na	—	—					
Pex22p	AAC04978	CAG60970	AAS52329	CAG97800	EAK91040	CAG88727	AAD45664	DQ384616	CAG77876					
Pex22p-like	—	—	—	—	—	na	—	—	EAL90994					
Pex26p	—	—	—	—	EAK91093	CAG88929	na	DQ645588	Antisense translation of NC_006072, nucleotides 117230- 118387					

	<i>Aspergillus fumigatus</i>	<i>Aspergillus nidulans</i>	<i>Penicillium chrysogenum</i>	<i>Magnaporthe grisea</i>	<i>Neurospora crassa</i>	<i>Gibberella zeae</i>	<i>Ustilago maydis</i>	<i>Cryptococcus neoformans</i> var. <i>neoformans</i>	<i>Schizosaccharomyces pombe</i>
Pex1p	EAL93310	EAA57740	AAG09748	XP_364454	EAA34641	EAA76787	EAK85195	AAW43248	CAA19256
Pex2p	EAL88068	EAA58944	DQ793192	XP_368589	EAA35361	EAA70670	EAK81310	AAW40683	CAA16981
Pex3p	EAL91965	EAA64392	DQ793193	XP_369909	EAA33751	EAA76989	EAK87104	AAW42444	CAB10141
Pex3Bp	—	—	—	—	—	—	—	—	—
Pex4p	EAL87211	Translation of AACD0-1000130, nucleotides 150195-150738 (contains intron)	DQ793194	XP_369064	EAA34737	EAA76379	Translation of AACP0-1000006, nucleotides 97041-96550 (contains intron)	—	CAB91184
Pex5p	EAL85289	EAA63772	AAR12222	XP_360528	EAA36111	EAA68640	EAK83659	AAW46349	CAA22179
Pex5Bp	—	—	—	—	—	—	—	—	—
Pex5Cp	—	—	—	—	—	—	—	—	—
Pex5/20p	—	—	—	—	—	—	EAK82973	AAW41849	—
Pex5Rp	—	—	—	—	—	—	—	—	—
Pex6p	EAL92776	EAA63496	AAG09749	XP_368715	EAA36040	EAA73732	EAK83459	AAW45333	CAB11501
Pex7p	EAL90870	EAA65909	DQ793195	XP_363555	AAN39560	EAA74171	EAK84499	AAW41119	P78798
Pex8p	EAL93137	EAA57947	DQ793196	XP_359449	EAA27783	EAA77627	EAK83936	AAW43468	CAB53406
Pex9p	—	—	—	—	—	—	—	—	—
Pex10p	EAL87045	EAA62774	DQ793197	XP_369099	EAA34967	EAA76761	EAK83811	AAW45079	CAB51769
Pex12p	EAL93972	EAA61357	DQ793198	XP_363845	EAA32773	EAA76413	EAK81282	AAW46724	CAD27496
Pex13p	EAL85282	EAA63824	DQ793199	XP_369087	EAA35785	EAA68396	EAK84395	AAW42381	CAB16740
Pex14p	EAL92562	EAA61046	DQ793200	XP_368216	EAA28304	EAA76904	EAK83123	AAW46857	CAA18656
Pex15p	—	—	—	—	—	—	—	—	—

Pex16p	EAL88469	EAA62294	DQ793201	XP_364166	EAA34648	EAA71849	EAK82801	AAW43797	CAA22819
Pex17p	See Pex14/17p	—	—	—	—	—	—	—	—
Pex14/17p	EAL93590	EAA58642	DQ793202	XP_368163	EAA27748	EAA73655	EAK81127	—	—
Pex18p	—	—	—	—	—	—	—	—	—
Pex19p	EAL92487	EAA60977	DQ793203	XP_368273	EAA31855	EAA70162	EAK86072	AAW42876	CAA97344
Pex20p	EAL90176	EAA60479	DQ793204	XP_368606	AAN39561	EAA76911	—	—	—
Pex21p	—	—	—	—	—	—	—	—	—
Pex21Bp	—	—	—	—	—	—	—	—	—
Pex22p	—	—	—	—	—	—	—	—	—
Pex22p-like	EAL90994	EAA66006	DQ793205	XP_365689	EAA26537	Translation of AACM0- 1000080, nucleotides 4362-3039 (contains intron)	—	—	—
Pex26p	EAL93994	EAA61336	DQ793206	XP_359606	EAA28582	EAA76391	—	—	—

* Partial ORFs encoded on non-overlapping contigs.

Mutations of Pex genes leading to impaired peroxisome biogenesis result in severe metabolic and developmental disturbances in yeasts, human and plants (Eckert, J. H. and R. Erdmann, *Rev. Physiol. Biochem Pharmacol* 147:75-121 (2003); Weller, S. et al., *Annual Review of Genomics and Human Genetics*, 4:165-211 (2003); Wanders, R. J., *Am. J. Med. Genet.*, 126A:355-375 (2004); Mano, S. and M. Nishimura, *Vitam Horm.*, 72:111-154 (2005); Wanders, J. A., and H. R. Waterham, *Annu. Rev. Biochem.*, 75:295-332 (2006); Fujiki, Yukio. Peroxisome Biogenesis Disorders. In, Encyclopedia of Life Sciences. John Wiley & Sons, 2006). For example, X-linked
 5
 10 adrenoleukodystrophy ["X-ALD"] and Zellweger syndrome, as well as several less severe forms of the disease, can result from single enzyme deficiencies and/or peroxisomal biogenesis disorders.

Within the yeast, *Yarrowia lipolytica*, a variety of different Pex genes have been isolated and characterized, as identified in Table 4 above. More
 15 specifically, Bascom, R.A. et al. (*Mol. Biol. Cell*, 14:939-957 (2003)) describe YIPex3p; Szilard, R. K. et al. (*J. Cell Biol.*, 131:1453-1469 (1995)) describe YIPex5p; Nuttley, W. M. et al. (*J. Biol. Chem.*, 269:556-566 (1994)) describe YIPex6p; Elizen G. A., et al. (*J. Biol. Chem.*, 270:1429-1436 (1995)) describe YIPex9p; Elizen G. A., et al. (*J. Cell Biol.*, 137:1265-1278 (1997)) and
 20 Titorenko, V. I. et al. (*Mol. Cell Biol.*, 17:5210-5226 (1997)) describe YIPex16p; Lambkin, G.R. and R.A. Rachubinski (*Mol. Biol. Cell.*, 12(11):3353-3364 (2001)) describe YIPex19; and Titorenko V.I., et al. (*J. Cell Biol.*, 142:403-420 (1998)) and Smith J.J. and R.A. Rachubinski (*J. Cell Biol.* 276:1618-1625 (2001)) describe YIPex20p.

25 Of initial interest herein was YIPex10p (GenBank Accession No. CAG81606, No. AB036770 and No. AJ012084). It was demonstrated in Sumita et al. (*FEMS Microbiol. Lett.*, 214:31-38 (2002)) that: 1) YIPex10p functions as a component of the peroxisome; and, 2) the C₃HC₄ zinc ring finger motif of YIPex10p was essential for the protein's function as

determined via creation of C341S, C346S and H343W point mutations, followed by analysis of growth.

Studies of the C₃HC₄ zinc ring finger motif of Pex10 have been done in other organisms with similar results. For example, point mutations that alter conserved residues in the Pex10p C₃HC₄ motif of *Pichia pastoris* were found to abolish function of the protein (Kalish, J. E. et al., *Mol. Cell Biol.*, 15:6406-6419 (1995)). Similarly, after functional complementation assays in fibroblast cell lines, Warren D. S., et al. (*Hum. Mutat.*, 15(6):509-521 (2000)) concluded that the C₃HC₄ motif was critical for Pex10p function. Several studies show that loss of function of Pex10p in *Arabidopsis* causes embryo lethality at the heart stage (Hu, J., et al., *Science*, 297:405-409 (2002); Schmummann, U. et al., *Proc. Natl. Acad. Sci. U.S.A.*, 100:9626-9631 (2003); Sparkes, I.A., et al., *Plant Physiol.*, 133:1809-1819 (2003); Fan, J. et al., *Plant Physiol.*, 139:231-239 (2005)). In follow-up research, Schemann, U. et al. (*Proc. Natl. Acad. Sci. U.S.A.*, 104:1069-1074 (2007)) investigated the function of Pex10p in nonlethal partial loss-of-function *Arabidopsis* mutants. Specifically, four T-DNA insertion lines expressing Pex10p with a dysfunctional C₃HC₄ motif were created in an *Arabidopsis* wildtype background. Mutant plants demonstrated impaired leaf peroxisomes and the authors suggest that inactivation of the ring finger motif in Pex10p eliminated protein interaction required for attachment of peroxisomes to chloroplasts and movement of metabolites between peroxisomes and chloroplasts.

Although studies have not identified essential domains in other Pex proteins, research has looked at the effect of various Pex mutants to learn the strategies and the molecular mechanisms evolutionarily diverse organisms use for assembling, maintaining, propagating and inheriting the peroxisome, an organelle known for its role in lipid metabolism. For example, Bascom, R.A. et al. has performed knockout and overexpression of the *Yarrowia lipolytica* Pex3p (*Mol. Biol. Cell*, 14:939-957 (2003)). The knockout cells did not contain wildtype peroxisomes but instead had numerous small vesicles;

overexpression resulted in cells with fewer, larger and clustered peroxisomes. They hypothesized that Pex3p is involved in the initiation of peroxisome assembly by sequestering components of peroxisome biogenesis, i.e., peroxisome targeting signal (PTS) 1 and 2 import machineries. Similarly, for
5 Guo, T. et al., knockout of the *Y. lipolytica* Pex16p resulted in excessive proliferation of immature peroxisomal vesicles and significantly decreased the rate and efficiency of their conversion to mature peroxisomes (*J. Cell Biol.*, 162:1255-1266 (2003)), while overexpression resulted in few but enlarged peroxisomes (Eitzen et al., *J. Cell Biol.*, 137:1265-1278 (1997)).
10 Guo et al. concluded Pex16p negatively regulated the membrane scission event required for division of early peroxisomal precursors.

Despite the advances summarized above, many details concerning the roles of various Pex proteins, their interaction with one another and the biogenesis/assembly mechanism in peroxisomes remains to be elucidated.
15 As such, the data described in the Application, wherein mutation within the C₃HC₄ motif of YIPex10p or knockout of YIPex3p, YIPex10p or YIPex16p results in creation of a *Yarrowia lipolytica* mutant that has an increased capacity to incorporate PUFAs, especially long-chain PUFAs such as C₂₀ to C₂₂ molecules, into the total lipid fraction and in the oil fraction in the cell, is a
20 novel observation that does not yet find validation in studies with other plants or animals.

It has been suggested that peroxisomes are required for both catabolic and anabolic lipid metabolism (Lin, Y. et al., *Plant Physiology*, 135:814-827 (2004)); however, this hypothesis was based on studies with a homolog of
25 Pex16p. More specifically, Lin, Y. et al. (*supra*) reported that Arabidopsis *Shrunken Seed 1* (*sse1*) mutants had both abnormal peroxisome biogenesis and fatty acid synthesis, based on a reduction of oil to approximately 10-16% of wild type in *sse1* seeds. Binns, D. et al. (*J. Cell Biol.*, 173(5):719-731 (2006)) examined the peroxisome-lipid body interactions in *Saccharomyces*
30 *cerevisiae* and determined that extensive physical contact between the two

organelles promotes coupling of lipolysis within lipid bodies with peroxisomal fatty acid oxidation. More specifically, ratios of free fatty acids to TAGs were examined in various Pex knockouts and found to be increased relative to the wildtype. Clearly, further investigation will be necessary to understand the metabolic roles of peroxisomes and in particular of Pex3p, Pex10p and Pex16p proteins.

Without wishing to be held to any particular explanation or theory, it is hypothesized that disruption or knockout of a Pex gene within an oleaginous yeast cell affects both the catabolic and anabolic lipid metabolism that naturally occurs in peroxisomes or is affected by peroxisomes. Disruption or knockout results in an increase in the amount of PUFAs in the total lipid fraction and in the oil fraction, as a percent of total fatty acids, as compared with an oleaginous yeast whose native peroxisome biogenesis factor protein has not been disrupted. In some cases, an increase in the amount of PUFAs in the total lipid fraction and in the oil fraction as a percent of dry cell weight, and/or an increase in the total lipid content as a percent of dry cell weight, is also observed. It is hypothesized that this generalized mechanism is applicable within all eukaryotic organisms, such as algae, fungi, oomycetes, yeast, euglenoids, stramenopiles, plants and some mammalian systems, since all comprise peroxisomes.

Identification And Isolation Of Pex Homologs

When the sequence of a particular Pex gene or protein within a preferred host organism is not known, one skilled in the art recognizes that it will be most desirable to identify and isolate these genes, or portions of them prior to regulating the activity of the encoded proteins, which regulation in turn facilitates altering the amount, as a percent of total fatty acids, of PUFAs incorporated into the total lipid fraction and in the oil fraction of the eukaryote. Sequence knowledge of the preferred host's Pex genes also facilitates disruption of the homologous chromosomal genes by targeted disruption.

The Pex sequences in Table 4, or portions of them, may be used to search for Pex homologs in the same or other algal, fungal, oomycete, euglenoid, stramenopiles, yeast 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. Use of software algorithms, such as the BLASTP method of alignment with a low complexity filter and the following parameters: Expect value = 10, matrix = Blosum 62 (Altschul, et al., *Nucleic Acids Res.* 25:3389-3402 (1997)), is well-known for comparing any Pex protein in Table 4 against a database of nucleic or protein sequences and thereby identifying similar known sequences within a preferred host organism.

Use of a software algorithm to comb through databases of known sequences is particularly suitable for the isolation of homologs having a relatively low percent identity to publicly available Pex sequences, such as those described in Table 4. It is predictable that isolation would be relatively easier for Pex homologs of at least about 70%-85% identity to publicly available Pex sequences. Further, those sequences that are at least about 85%-90% identical would be particularly suitable for isolation and those sequences that are at least about 90%-95% identical would be the most facilely isolated.

Some Pex homologs have also been isolated by the use of motifs unique to the Pex enzymes. For example, it is well known that Pex2p, Pex10p and Pex12p all share a cysteine-rich motif near their carboxyl terminus known as a C₃HC₄ zinc ring finger motif (FIG. 2A). This region of “conserved domain” corresponds to a set of amino acids that are highly conserved at specific positions and likely represents a region of the Pex protein that is essential to the structure, stability or activity of the protein. Motifs are identified by their high degree of conservation in aligned sequences of a family of protein homologues. As unique “signatures”, they can determine if a protein with a newly determined sequence belongs to a previously identified

protein family. These motifs are useful as diagnostic tools for the rapid identification of novel Pex2, Pex10 and/or Pex12 genes, respectively.

Alternatively, the publicly available Pex sequences or their motifs may be hybridization reagents for the identification of 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 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 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 known. Typically the probe and the sample must be mixed under conditions that 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 the sample nucleic acid occurs. The concentration of probe or target in the mixture determine the time necessary for hybridization to occur. The higher the concentration of the probe or target, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added, such as guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate,

potassium iodide or cesium trifluoroacetate. If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v) ["by volume"].

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 are unlabeled carrier nucleic acids from about 0.1 to 5 mg/mL, fragmented nucleic DNA such as calf thymus or salmon sperm DNA or yeast RNA, and optionally from about 0.5 to 2% wt/vol ["weight by volume"] glycine. Other additives may be included, such as volume exclusion agents that include polar water-soluble or swellable agents (e.g., polyethylene glycol), anionic polymers (e.g., polyacrylate or polymethylacrylate) and anionic saccharidic polymers, such as 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 or covalently coupled to it immobilized nucleic acid probe that is unlabeled and complementary to one portion of the sequence.

Any of the Pex nucleic acid fragments or any identified homologs may be used to isolate genes encoding homologous proteins from the same or other algal, fungal, oomycete, euglenoid, stramenopiles, yeast 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, such as polymerase chain reaction ["PCR"] (U.S. Pat. No.4,683,202); ligase chain reaction ["LCR"] (Tabor, S. et al., *Proc. Natl. 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 proteins or polypeptides similar to publicly available Pex genes or their motifs could be isolated directly by using all or a portion of those publicly available nucleic acid fragments as DNA hybridization probes to screen libraries from any desired organism using well known methods. Specific oligonucleotide probes based upon the publicly available 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, such as 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 or the full length of the publicly available sequences or their motifs. 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 (Thein and Wallace, "The use of oligonucleotides 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; Rychlik, W.,

In Methods in Molecular Biology, White, B. A. Ed., (1993) Vol. 15, pp 31-39, PCR Protocols: Current Methods and Applications. Humana: Totowa, NJ).

Generally two short segments of available Pex 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 available nucleic acid fragments or their motifs. 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 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. Natl. 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 available sequences. Using commercially available 3' RACE or 5' RACE systems (e.g BRL, Gaithersburg, MD), specific 3' or 5' cDNA fragments can be isolated (Ohara et al., *Proc. Natl. Acad. Sci. U.S.A.*, 86:5673 (1989); Loh et al., *Science*, 243:217 (1989)).

Based on any of these well-known methods just discussed, it would be possible to identify and/or isolate Pex gene homologs in any preferred eukaryotic organism of choice. The activity of any putative Pex gene can readily be confirmed by targeted disruption of the endogenous gene within the PUFA-producing host organism, since the lipid profiles of the total lipid fraction and of the oil fraction are modified relative to those within an organism lacking the targeted Pex gene disruption.

Increasing The Amount Of PUFAs In The Total Lipid Fraction And In The Oil Fraction Via Disruption Of A Native Peroxisome Biogenesis Factor Protein

As noted above, the present disclosure relates to the following described methods for increasing the weight percent of one PUFA or a combination of PUFAs in an oleaginous eukaryotic organism, comprising:

- a) providing an oleaginous eukaryotic organism comprising a
5 disruption in a native gene encoding a peroxisome biogenesis factor protein, which creates a PEX-disruption organism; and genes encoding a functional PUFA biosynthetic pathway; and,
 - b) growing the eukaryotic organism of (a) under conditions wherein the
10 weight percent of one PUFA or a combination of PUFAs is increased in the total lipid fraction and in the oil fraction relative to the weight percent of the total fatty acids, when compared with those weight percents in an oleaginous eukaryotic organism whose native peroxisome biogenesis factor protein has not been disrupted.
- 15 The amount of PUFAs that increases as a percent of total fatty acids can be: 1) the PUFA that is the desired end product of a functional PUFA biosynthetic pathway, as opposed to PUFA intermediates or by-products; 2) C₂₀ to C₂₂ PUFAs; and/or, 3) total PUFAs.

In addition to the increase in the weight percent of one or a
20 combination of PUFAs relative to the weight percent of the total fatty acids, in some cases, the total lipid content (TFA % DCW) of the cell may be increased or decreased. What this means is that regardless of whether the disruption in the PEX gene causes the amount of total lipids in the PEX-disrupted cell to increase or decrease, the disruption always causes the
25 weight percent of a PUFA or of a combination of PUFAs to increase.

Another method provided herein relates to a disruption in a native gene encoding a peroxisome biogenesis factor protein, wherein said disruption can result in an increase in the percent of one PUFA or a combination of PUFAs relative to the dry cell weight when compared to that
30 percent in a parental strain whose native Pex protein had not been disrupted

or that was expressing a “replacement” copy of the disrupted native Pex protein.

In preferred aspects of the method above, the disruption in a native gene encoding a peroxisome biogenesis factor protein results in an increase
5 in the amount of the PUFA that is the desired end product of a functional PUFA biosynthetic pathway, as opposed to PUFA intermediates or by-products, as a percent of dry cell weight relative to the parental strain whose native Pex protein had not been disrupted or the parental strain that was expressing a “replacement” copy of the disrupted native Pex protein. In some
10 cases, the increase in the percent of a combination of PUFAs relative to the dry cell weight is a combination of C₂₀ to C₂₂ PUFAs or the total PUFAs.

Also described herein are organisms produced by these methods, comprising a disruption of at least one peroxisome biogenesis factor protein. Lipids and oils obtained from these organisms, products obtained from the
15 processing of the lipids and oil, use of these lipids and oil in foods, animal feeds or industrial applications and/or use of the by-products in foods or animal feeds are also described.

Preferred eukaryotic organisms in the methods described above include algae, fungi, oomycetes, yeast, euglenoids, stramenopiles, plants and
20 some mammalian systems.

The peroxisome biogenesis factor protein for any of these methods may be selected from the group consisting of: Pex1p, Pex2p, Pex3p, Pex3Bp, Pex4p, Pex5p, Pex5Bp, Pex5Cp, Pex5/20p, Pex6p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex15p, Pex16p, Pex17p, Pex14/17p, Pex18p,
25 Pex19p, Pex20p, Pex21p, Pex21B, Pex22p, Pex22p-like and Pex26p (and protein homologs thereof). In some preferred methods described herein, the disrupted peroxisome biogenesis factor protein is selected from the group consisting of: Pex2p, Pex3p, Pex10p, Pex12p and/or Pex16p. In some more preferred methods, however, the disrupted peroxisome biogenesis factor

protein is selected from the group consisting of: Pex3p, Pex10p and/or Pex16p.

The disruption in the native gene encoding a peroxisome biogenesis factor protein can be an insertion, deletion, or targeted mutation within a portion of the gene, such as within the N-terminal portion of the protein or within the C-terminal portion of the protein. Alternatively, the disruption can result in a complete gene knockout such that the gene is eliminated from the host cell genome. Or, the disruption could be a targeted mutation that results in a non-functional protein.

10 Disruption Methodologies

The invention includes disruption in a native gene encoding a peroxisome biogenesis factor protein within a preferred host cell. Although numerous techniques are available to one of skill in the art to achieve disruption, generally the endogenous activity of a particular gene can be reduced or eliminated by the following techniques, for example: 1) disrupting the gene through insertion, substitution and/or deletion of all or part of the target gene; or 2) manipulating the regulatory sequences controlling the expression of the protein. Both of these techniques are discussed below. However, one skilled in the art appreciates that these are well described in the existing literature and are not limiting to the methods, host cells, and products described herein. One skilled in the art also appreciates the most appropriate technique for use with any particular oleaginous yeast.

Disruption Via Insertion, Substitution And/Or Deletion: For gene disruption, a foreign DNA fragment, typically a selectable marker gene, is inserted into the structural gene. This interrupts the coding sequence of the structural gene and causes inactivation of that gene. Transformation of the disruption cassette into the host cell results in replacement of the functional native gene by homologous recombination with the non-functional disrupted gene. See, for example: Hamilton et al., *J. Bacteriol.*, 171:4617-4622 (1989); Balbas et al., *Gene*, 136:211-213 (1993); Gueldener

et al., *Nucleic Acids Res.*, 24:2519-2524 (1996); and Smith et al., *Methods Mol. Cell. Biol.*, 5:270-277(1996). One skilled in the art appreciates the many variations of the general method of gene targeting, which admits of positive c
negative selection, creation of gene knockouts, and insertion of exogenous
5 DNA sequences into specific genome sites in mammalian systems, plant cells, filamentous fungi, algae, oomycetes, euglenoids, stramenopiles, yeast and/or microbial systems.

In contrast, a non-specific method of gene disruption is the use of transposable elements or transposons. Transposons are genetic elements
10 that insert randomly into DNA but can be later retrieved on the basis of sequence to determine the locus of insertion. Both *in vivo* and *in vitro* transposition techniques are known and involve the use of a transposable element in combination with a transposase enzyme. When the transposable element or transposon is contacted with a nucleic acid fragment in the
15 presence of the transposase, the transposable element randomly inserts into the nucleic acid fragment. The technique is useful for random mutagenesis and for gene isolation, since the disrupted gene may be identified on the basis of the sequence of the transposable element. Kits for *in vitro* transposition are commercially available and include: the Primer Island Transposition Kit,
20 available from Perkin Elmer Applied Biosystems, Branchburg, NJ, based upon the yeast Ty1 element; the Genome Priming System, available from New England Biolabs, Beverly, MA, based upon the bacterial transposon Tn7; and EZ::TN Transposon Insertion Systems, available from Epicentre Technologies, Madison, WI, based upon the Tn5 bacterial transposable element.

25 **Manipulation Of Pex Regulatory Sequences:** As is well known in the art, the regulatory sequences associated with a coding sequence include transcriptional and translational "control" nucleotide sequences located upstream (5' non-coding sequences), within, or downstream (3' non-coding sequences) of the coding sequence, and which influence the transcription,
30 RNA processing or stability, or translation of the associated coding sequence

Thus, manipulation of a Pex gene's regulatory sequences may refer to manipulation of the promoters, silencers, 5' untranslated leader sequences (between the transcription start site and the translation initiation codon), introns, enhancers, initiation control regions, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures of the particular Pex gene. In all cases, however, the result of the manipulation is down-regulation of the Pex gene's expression, which promotes increased amount of PUFAs in the total lipid fraction and in the oil fraction, as a percent of total fatty acids, as compared with an oleaginous yeast whose native peroxisome biogenesis factor protein has not been disrupted.

For example, the promoter of a Pex10 gene could be deleted or disrupted. Alternatively, the native promoter driving expression of a Pex10 gene may be substituted with a heterologous promoter having diminished promoter activity with respect to that of the native promoter. Methods useful for manipulating regulatory sequences are well known.

The skilled person is able to use these and other well known techniques to disrupt a native peroxisome biogenesis factor protein within the preferred host cells described herein, such as mammalian systems, plant cells, filamentous fungi, algae, oomycetes, euglenoids, stramenopiles and yeast.

One skilled in the art is able to discern the optimum means to disrupt the native Pex gene to achieve an increased amount of PUFAs that accumulate in the total lipid fraction and in the oil fraction, as a percent of total fatty acids, as compared with a eukaryotic organisms whose native peroxisome biogenesis factor protein has not been disrupted.

Metabolic Engineering Of ω -3 And/Or ω -6 Fatty Acid Biosynthesis

In addition to the methods described herein for disruption of a native peroxisome biogenesis factor protein, it may also be useful to manipulate ω -3 and/or ω -6 fatty acid biosynthesis. This may require metabolic engineering

directly within the PUFA biosynthetic pathway or additional manipulation of pathways that contribute carbon to the PUFA biosynthetic pathway.

Techniques useful for up-regulating desirable biochemical pathways and down-regulating undesirable biochemical pathways are well known 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, such as antisense mRNA and zinc-finger targeting technologies.

The following discuss altering the PUFA biosynthetic pathway as a means to increase GLA, ARA, EPA or DHA, respectively, and desirable manipulations in the TAG biosynthetic pathway and in the TAG degradation pathway: Int'l. App. Pub. No. WO 2006/033723, Int'l. App. Pub. No. WO 2006/055322 [U.S. Pat. Appl. Pub. No. 2006-0094092-A1], Int'l. App. Pub. No. WO 2006/052870 [U.S. Pat. Appl. Pub. No. 2006-0115881-A1] and Int'l. App. Pub. No. WO 2006/052871 [U.S. Pat. Appl. Pub. No. 2006-0110806-A1], respectively.

Expression Systems, Cassettes, Vectors And Transformation Of Host Cells

It may be necessary to create and introduce a recombinant construct into the preferred eukaryotic host, such as e.g., mammalian systems, plant cells, filamentous fungi, algae, oomycetes, euglenoids, stramenopiles and yeast, to result in disruption of a native peroxisome biogenesis factor protein and/or introduction of genes encoding a PUFA biosynthetic pathway. One of skill in the art appreciates standard resource materials that describe: 1) specific conditions and procedures for construction, manipulation and isolation of macromolecules, such as DNA molecules, plasmids, etc.; 2) generation of recombinant DNA fragments and recombinant expression constructs; and 3) screening and isolating of clones. See Sambrook et al., Molecular Cloning: A Laboratory Manual, 2nd ed., Cold Spring Harbor

Laboratory: Cold Spring Harbor, NY (1989); Maliga et al., Methods in Plant Molecular Biology, Cold Spring Harbor, NY (1995); Birren et al., Genome Analysis: Detecting Genes, v. 1, Cold Spring Harbor, NY (1998); Birren et al. Genome Analysis: Analyzing DNA, v. 2, Cold Spring Harbor: NY (1998); Plar
5 Molecular Biology: A Laboratory Manual, Clark, ed. Springer: NY (1997).

In general, the choice of sequences included in the construct depends on the desired expression products, the nature of the host cell and the proposed means of separating transformed cells versus non-transformed cells. The skilled artisan is well aware of the genetic elements that must be
10 present on the plasmid vector to successfully transform, select and propagate host cells containing the chimeric gene. Typically, however, the vector or cassette contains sequences directing transcription and translation of the relevant gene(s), a selectable marker and sequences allowing autonomous replication or chromosomal integration. Suitable vectors comprise a region
15 of the gene that controls transcriptional initiation, i.e., a promoter, 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 host cell.

Initiation control regions or promoters useful for driving expression of
20 heterologous genes or portions of them in the desired host cell are numerous and well known. These control regions may comprise a promoter, enhancer, silencer, intron sequences, 3' UTR and/or 5' UTR regions, and protein and/or RNA stabilizing elements. Such elements may vary in their strength and specificity. Virtually any promoter (i.e., native, synthetic, or chimeric) capable
25 of directing expression of these genes in the selected host cell is suitable. Expression in a host cell can occur in an induced or constitutive fashion. Induced expression occurs by inducing the activity of a regulatable promoter operably linked to the Pex gene of interest. Constitutive expression occurs by the use of a constitutive promoter operably linked to the gene of interest.

When the host cell is, for example, yeast, transcriptional and translational regions functional in yeast cells are provided, particularly from the host species. See Int'l. App. Pub. No. WO 2006/052870 for preferred transcriptional initiation regulatory regions for use in *Yarrowia lipolytica*. Any
5 of a number of regulatory sequences may be used, depending on whether constitutive or induced transcription is desired, the efficiency of the promoter in expressing the ORF of interest, the ease of construction, etc.

3' non-coding sequences encoding transcription termination signals, i.e., a "termination region", must be provided in a recombinant construct and
10 may be 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 in both the same and different genera and species from which they were derived. The termination region is selected more for convenience rather than for any
15 particular property. Termination regions may also be derived from various genes native to the preferred hosts.

Particularly useful termination regions for use in yeast are those derived from a yeast gene, particularly *Saccharomyces*,
Schizosaccharomyces, *Candida*, *Yarrowia* or *Kluyveromyces*. The 3'-regions
20 of mammalian genes encoding γ -interferon and α -2 interferon are also known to function in yeast. 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. A termination region may be unnecessary, but is highly preferred.

25 The vector may comprise a selectable and/or scorable marker, in addition to the regulatory elements described above. Preferably, the marker gene is an antibiotic resistance gene such that treating cells with the antibiotic causes inhibition of growth, or death, of untransformed cells and uninhibited growth of transformed cells. For selection of yeast transformants, any marker
30 that functions in yeast is useful with resistance to kanamycin, hygromycin and

the amino glycoside G418 and the ability to grow on media lacking uracil, lysine, histine or leucine being particularly useful.

Merely inserting a gene into a cloning vector does not ensure its expression at the desired rate, concentration, amount, etc. 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 transcription, RNA stability, translation, protein stability and location, oxygen limitation, and secretion from the host cell. Some of the manipulated features include: the nature of the relevant transcriptional promoter and terminator sequences, the number of copies of the cloned gene and 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 may be used in the methods and host cells described herein to further optimize expression of PUFA biosynthetic pathway genes and to diminish expression of a native Pex gene.

After a recombinant construct is created, e.g., comprising a chimeric gene comprising a promoter, ORF and terminator, suitable for disruption or knock out of a native peroxisome biogenesis factor protein and/or expression of genes encoding a PUFA biosynthetic pathway activity, it is placed in a plasmid vector capable of autonomous replication in the host cell or 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 with 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.

When two or more genes are expressed from separate replicating vectors, each vector may have a different means of selection and should lack
5 homologous 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
10 products.

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

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence, for example, in an expression cassette, is referred to herein as "transformed" or "recombinant". The transformed host
20 will have at least one copy of the expression construct and may have two or more, depending upon whether the gene is integrated into the genome, amplified, or is present on an extrachromosomal element having multiple copy numbers.

The transformed host cell can be identified by selection for a marker
25 contained on the introduced construct. Alternatively, a separate marker construct may be co-transformed with the desired construct, as many transformation techniques introduce many DNA molecules into host cells. Typically, transformed hosts are selected for their ability to grow on selective media. Selective media may incorporate an antibiotic or lack a factor
30 necessary for growth of the untransformed host, such as a nutrient or growth

factor. An introduced marker gene may confer antibiotic resistance, or encode an essential growth factor or enzyme, thereby permitting growth on selective media when expressed in the transformed host. Selection of a transformed host can also occur when the expressed marker protein can be detected, either directly or indirectly. The marker protein may be expressed alone or as a fusion to another protein. The marker protein can be detected by its enzymatic activity (e.g., β -galactosidase can convert the substrate X-gal ["5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside"] to a colored product; luciferase can convert luciferin to a light-emitting product) or its light-producing or modifying characteristics (e.g., the green fluorescent protein of *Aequorea victoria* fluoresces when illuminated with blue light). Alternatively, antibodies can be used to detect the marker protein or a molecular tag on, for example, a protein of interest. Cells expressing the marker protein or tag can be selected, for example, visually, or by techniques such as fluorescence-activated cell sorting or panning using antibodies.

Regardless of the selected host or expression construct, multiple transformants must be screened to obtain a strain or plant line displaying the desired expression level, regulation and pattern, as different independent transformation events 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)). Such screening may be accomplished by Southern analysis of DNA blots (Southern, *J. Mol. Biol.*, 98:503 (1975)), Northern analysis of mRNA expression (Kroczeck, *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.

Preferred Eukaryotic Host Organisms

A variety of eukaryotic organisms are suitable as host herein, to thereby yield a transformant host organism comprising a disruption in a native peroxisome biogenesis factor protein and genes encoding a PUFA biosynthetic pathway, wherein the transformed eukaryotic host organism has

an increased amount of PUFAs incorporated into the total lipid fraction and in the oil fraction, as a percent of total fatty acids, as compared to a eukaryotic organism whose native peroxisome biogenesis factor protein has not been disrupted. Various mammalian systems, plant cells, fungi, algae, oomycetes
5 yeasts, stramenopiles and/or euglenoids may be useful hosts. Although oleaginous organisms are preferred, non-oleaginous organisms also have utility herein such that, when one of their native PEX genes is disrupted, an increase in the weight percent of at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in the total lipid fraction or in
10 the oil fraction will be experienced and may lead to a 1.3 fold increase in the PUFA. Additionally, the percent of the PUFA may be increased relative to the dry cell weight in the non-oleaginous organism. In alternate embodiments, a non-oleaginous organism can be genetically modified to become oleaginous, e.g., yeast such as *Saccharomyces cerevisiae*.

15 Oleaginous organisms are naturally capable of oil synthesis and accumulation, wherein the total oil content typically comprises greater than about 25% of the cellular dry weight. Various algae, moss, fungi, yeast, stramenopiles and plants are naturally classified as oleaginous.

Preferred oleaginous microbes include those algal, stramenopile and
20 fungal organisms that naturally produce ω -3/ ω -6 PUFAs. For example, ARA, EPA and/or DHA is produced via *Cyclotella* sp., *Nitzschia* sp., *Pythium*, *Thraustochytrium* sp., *Schizochytrium* sp. and *Mortierella*. The method of transformation of *M. alpina* is described by Mackenzie et al. (*Appl. Environ. Microbiol.*, 66:4655 (2000)). Similarly, methods for transformation of
25 Thraustochytriales microorganisms (e.g., *Thraustochytrium*, *Schizochytrium*) are disclosed in U.S. Pat. No. 7,001,772.

More preferred are oleaginous yeast, including those that naturally produce and those genetically engineered to produce ω -3/ ω -6 PUFAs. Genera typically identified as oleaginous yeast include, but are not limited to:
30 *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*,

Trichosporon and *Lipomyces*. More specifically, illustrative oil-synthesizing yeasts include: *Rhodospiridium 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*).

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

Specific teachings relating to transformation of *Yarrowia lipolytica* include U.S. Pat. No. 4,880,741 and U.S. Pat. No. 5,071,764 and Chen, D. C et al. (*Appl. Microbiol. Biotechnol.*, 48(2):232-235 (1997)), while suitable selection techniques are described in U.S. Pat. No. 7,238,482 and Int'l. App. Pub. Nos. WO 2005/003310 and WO 2006/052870.

The preferred method of expressing genes in *Yarrowia lipolytica* is by integration of linear DNA into the genome of the host. Integration into multiple locations within the genome can be particularly useful when high level expression of genes are desired, such as 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 $\Delta 12$ desaturase gene locus (U.S. Pat. No. 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), the *Pex3* gene locus (GenBank Accession No. CAG78565), the *Pex16* gene locus (GenBank Accession No. CAG79622) 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. 5-fluoroorotic acid [5-fluorouracil-6-carboxylic acid monohydrate or “5-FOA”] may also be used for selection of yeast *Ura⁻* mutants. This 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 Int'l. App. Pub. 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, i.e., W497L, that confers sulfonyl urea herbicide resistance (Int'l. App. Pub. 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.

Fermentation Processes For Polyunsaturated Fatty Acid Production

The transformed host cell is grown under conditions that optimize expression of PUFA biosynthetic genes and produce the greatest and most economical yield of desired PUFAs. In general, media conditions may be optimized by modifying 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. Oleaginous yeast of interest, such as *Yarrowia*

lipolytica, are generally grown in a complex medium such as yeast extract-peptone-dextrose broth (YPD) or a defined minimal media that lacks a component necessary for growth and forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, 5 Detroit, MI)).

Fermentation media for the methods and host cells described herein must contain a suitable carbon source such as are taught in U.S. Pat. No. 7,238,482. Suitable sources of carbon encompass a wide variety of sources with sugars, glycerol and/or fatty acids being preferred. Most preferred is 10 glucose and/or fatty acids containing between 10-22 carbons.

Nitrogen may be supplied from an inorganic (e.g., $(\text{NH}_4)_2\text{SO}_4$) 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 15 those skilled in the art suitable for the growth of the oleaginous yeast and the promotion of the enzymatic pathways of PUFA production. Particular attention is given to several metal ions, such as Fe^{+2} , Cu^{+2} , Mn^{+2} , Co^{+2} , Zn^{+2} and 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 for the methods and host cells described 20 herein 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 is well known in microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to 25 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 increased amounts of PUFAs and TAGs in 30 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 oils in oleaginous yeast. This approach is described in U.S.

Pat. No. 7,238,482, as are various suitable fermentation process designs

5 (i.e., batch, fed-batch and continuous) and considerations during growth.

Purification And Processing Of PUFA Oils

Fatty acids, including PUFAs, may be found in the host organisms as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids. These fatty acids may be extracted from the host

10 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)).

15 In general, means for the purification of fatty acids (including PUFAs) may include extraction (e.g., U.S. Pat. No. 6,797,303 and U.S. Pat. No. 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. See U.S. Pat. No. 7,238,482.

20 Oils For Use In Foodstuffs, Health Food Products, Pharmaceuticals And Animal Feeds

The market place contains many food and feed products, incorporating ω -3 and/or ω -6 fatty acids, particularly ALA, GLA, ARA, EPA, DPA and DHA.

It is contemplated that the microbial biomass comprising long-chain PUFAs,

25 partially purified microbial biomass comprising PUFAs, purified microbial oil comprising PUFAs, and/or purified PUFAs made by the methods and host cells described herein impart health benefits, upon ingestion of foods or feed improved by their addition. These oils can be added to food analogs, drinks meat products, cereal products, baked foods, snack foods and dairy products
30 to name a few. See U.S. Pat. App. Pub. No. 2006/0094092.

These compositions may impart health benefits by being added to medical foods including medical nutritionals, dietary supplements, infant formula and pharmaceuticals. The skilled artisan will appreciate the amount of the oils to be added to food, feed, dietary supplements, nutraceuticals, pharmaceuticals, and other ingestible products as to impart health benefits. Health benefits from ingestion of these oils are described in the art, known to the skilled artisan and continuously investigated. Such an amount is referred to herein as an “effective” amount and depends on, among other things, the nature of the ingested products containing these oils and the physical conditions they are intended to address.

DESCRIPTION OF PREFERRED EMBODIMENTS

As demonstrated in the Examples and summarized in Table 5, *infra*, disruptions in the C-terminal portion of the C₃HC₄ zinc ring finger motif of YIPex10p, deletion of the entire chromosomal YIPex10 gene or of the entire chromosomal YIPex16 gene, deletion of both the entire chromosomal YIPex10 and the YIPex16 gene, and deletion of the entire chromosomal YIPex3 gene all resulted in an engineered PUFA-producing strain of *Yarrowia lipolytica* that had an increased weight percent of PUFAs as a percent of total fatty acids, relative to the parental strain whose native Pex protein had no disruption. Expression of an extrachromosomal YIPex10p in an engineered EPA-producing strain of *Yarrowia lipolytica* that possessed a disruption in the genomic Pex10p and an increased amount of PUFAs in the total lipid fraction and in the oil fraction reversed the effect.

Table 5 compiles data from Examples 3, 4, 5, 7, 9, 11 and 12, such that trends concerning total lipid content [“TFAs % DCW”], concentration of a given fatty acid(s) expressed as a weight percent of total fatty acids [“% TFAs”], and content of a given fatty acid(s) as its percent of the dry cell weight [“% DCW”] can be deduced, based on the presence/absence of a Pex disruption or knockout. “Desired PUFA % TFAs” and “Desired PUFA % DCW” quantify the particular concentration or content, respectively, of the

desired PUFA product (i.e., DGLA or EPA) that the engineered PUFA biosynthetic pathway was designed to produce. "All PUFAs" includes LA, ALA, EDA, DGLA, ETrA, ETA and EPA, while "C20 PUFAs" is limited to EDA, DGLA, ETrA, ETA and EPA.

Table 5
PUFA % TFAs and % DCW In *Yarrowia lipolytica* Strains With Mutant Pex Genes

Example	Strain	Genomic Pex Gene	TFA % DCW	% TFAs			% DCW		
				Desired PUFA	All PUFAs	C20 PUFAs	Desired PUFA	All PUFAs	C20 PUFAs
3, 4	Y4086	Wildtype Pex10	28.6	9.8 [EPA]	60.1	25.2	2.8 [EPA]	17.2	7.2
	Y4128	Mutant* Pex10	11.2	42.8 [EPA]	79.3	57.9	4.8 [EPA]	8.9	6.4
5	Y4128U1 + pFBAIn- PEX10	Mutant* Pex10 + Plasmid Wildtype Pex10 within chimeric FBAInm::Pex10::Pex20 gene	29.2	10.8 [EPA]	60	27.3	3.1 [EPA]	17.5	8.0
	Y4128U1 + pPEX10-1	Mutant* Pex10 + Plasmid Wildtype Pex10 within Pex10-5' (500 bp):: Pex10::Pex10-3' gene	27.1	10.7 [EPA]	60.1	26.7	2.9 [EPA]	16.2	7.2
	Y4128U1 + pPEX10-2	Mutant* Pex10 + Plasmid Wildtype Pex10 within Pex10-5' (991 bp):: Pex10::Pex10-3' gene	28.5	10.8 [EPA]	59	26.9	3.1 [EPA]	16.8	7.7
7	Y4128U1 + control	Mutant* Pex10	22.8	27.7 [EPA]	62.6	42.3	6.3 [EPA]	14.2	9.6
	Y4184U	Wildtype Pex10	11.8	20.6 [EPA]	nq [♦]	nq [♦]	2.4 [EPA]	nq [♦]	nq [♦]
			8.8	23.2 [EPA]	nq [♦]	nq [♦]	2.0 [EPA]	nq [♦]	nq [♦]
	Y4184U	Mutant Pex10	17.6	43.2	nq [♦]	nq [♦]	7.6	nq [♦]	nq [♦]

	Δ Pex10			[EPA]			[EPA]		
		13.2		46.1 [EPA]	nq [♦]	nq [♦]	6.1 [EPA]	nq [♦]	nq [♦]
9	Y4036 (avg)	Nq [♦]	Wildtype Pex16	23.4 [DGLA]	61.5	33.7	nq [♦]	nq [♦]	nq [♦]
	Y4036 (Δ Pex16) (avg)	Nq [♦]	Mutant Pex16	43.4 [DGLA]	69.1	49.1	nq [♦]	nq [♦]	nq [♦]
11	Y4305U (Δ pex10) (avg)	30	Mutant Pex10 and Wildtype Pex16	44.7 [EPA]	76.6	55.4	13.4 [EPA]	23.0	16.6
	Y4305 (Δ Pex10, Δ Pex16) (avg)	30	Mutant Pex10, Mutant Pex16	48.3 [EPA]	79.0	57.7	14.5 [EPA]	23.7	17.3
12	Y4036	4.7	Wildtype Pex3	19 [DGLA]	57	27	0.9 [DGLA]	2.7	1.3
	Y4036 (Δ Pex3)	6.1	Mutant Pex3	46 [DGLA]	68	56	2.8 [DGLA]	4.4	3.4
		5.9		46 [DGLA]	68	56	2.7 [DGLA]	4.0	3.3

* Pex10 disruption in Y4128 results in a truncated protein, wherein the last 32 amino acids of the C-terminus (corresponding to the C-terminal portion of the C₃HC₄ zinc ring finger motif) are not present.

♦ nq = not quantified

Although data cannot be directly compared between Examples, as a result of different *Yarrowia* strains and growth conditions, the following conclusions can be drawn (relative to the parental strain whose native Pex protein had not been disrupted or the parental strain that was expressing a “replacement” copy of the disrupted native Pex protein):

- 1) Pex disruption in a PUFA-producing *Yarrowia* results in an increase in the weight percent of a single PUFA, for example EPA or DLGA, relative to the weight percent of total fatty acids (% TFAs) in the total lipid fraction and in the oil fraction;
- 2) Pex disruption in a PUFA-producing *Yarrowia* results in an increase in the weight percent of C₂₀ PUFAs relative to the weight percent of total fatty acids in the total lipid fraction and in the oil fraction;
- 3) By the extension of point 1), Pex disruption in a PUFA-producing *Yarrowia* results in an increase in the amount of any and all combinations of PUFAs relative to the weight percent of total fatty acids in the total lipid fraction and in the oil fraction; and
- 4) Pex disruption in a PUFA-producing *Yarrowia* results in an increase in the percent of a single PUFA, for example EPA or DLGA, relative to the dry cell weight.

Variable results are observed when comparing the effects of Pex disruptions in “All PUFAs % DCW”, “C₂₀ PUFAs % DCW” and TFA % DCW. Specifically, in some cases, the Pex disruption in the PUFA-producing *Yarrowia* results in an increased amount of C₂₀ PUFAs or All PUFAs, as a percent of dry cell weight, in the total lipid fraction and in the oil fraction (relative to the parental strain whose native Pex protein had not been disrupted). In other cases, there is a diminished amount of C₂₀ PUFAs or All PUFAs, as a percent of dry cell weight, in the total lipid fraction and in the oil fraction (relative to the parental strain whose native Pex protein had not been disrupted). Similar results are observed with respect to the total lipid content

(TFA % DCW), in that the effect of the Pex disruption can either result in an increase in total lipid content or a decrease.

Although each of the above generalizations are of interest, it is particularly useful to examine the effect of the Pex disruptions on the ratio of the desired PUFA which the organism was engineered to produce relative to the amount of total PUFAs.

For example, 54% of the PUFAs (as a % TFAs) were EPA in strain Y4128 containing the Pex10 disruption that resulted in truncation of the last 32 amino acids of the C-terminus, while only 16.3% of the PUFAs (as a % TFAs) were EPA in the parent strain, Y4086. Thus, the disruption was responsible for a 3.3-fold increase in the amount of the desired PUFA (as % TFAs) (Examples 3, 4). In a similar manner, 62.8% of the PUFAs (as a % TFAs) were DGLA in strain Y4036 (Δ Pex16), while only 38.1% the PUFAs (as a % TFAs) were DGLA in Y4036—a 1.65 fold increase (Example 9). And, 67.7% of the PUFAs (as a % TFAs) were DGLA in strain Y4036 (Δ Pex3), while only 33.3% the PUFAs (as a % TFAs) were DGLA in Y4036—a 2.0 fold increase (Example 12). These results support the hypothesis that the Pex disruption results in a selective increase in the amount, as a % TFAs, of the desired PUFA which the organism was engineered to produce in the total lipid and oil fractions.

Less significant selectivity is observed when examining the effect of Pex disruptions on the ratio of C20 PUFAs relative to the amount of total PUFAs. For example, 73% of the PUFAs (as a % TFAs) were C20 PUFAs in strain Y4128 containing the Pex10 disruption, while only 42% of the PUFAs (as a % TFAs) were C20 PUFAs in strain Y4086. Thus, the disruption was responsible for a 1.7-fold increase in the amount of C20 PUFAs that accumulated in the total lipid and oil fractions, relative to the total PUFAs (Examples 3, 4). In a similar manner, 71% of the PUFAs (as a % TFAs) were C20 PUFAs in strain Y4036 (Δ Pex16), while only 54.8% the PUFAs (as a % TFAs) were C20 PUFAs in Y4036—a 1.3 fold increase (Example 9). And,

82.4% of the PUFAs (as a % TFAs) were C20 PUFAs in strain Y4036 (Δ Pex3), while only 47.4% the PUFAs (as a % TFAs) were C20 PUFAs in Y4036—a 1.7 fold increase (Example 12).

On the basis of the teachings and results described herein, it is expected that the feasibility and commercial utility of utilizing various disruptions in native genes encoding peroxisome biogenesis factor proteins as a means to increase the amount of PUFAs produced in a PUFA-producing eukaryotic organism will be appreciated. The PUFA-producing eukaryotic organism can synthesize a variety of ω -3 and/or ω -6 PUFAs, using either the Δ 9 elongase/ Δ 8 desaturase pathway or the Δ 6 desaturase/ Δ 6 elongase pathway.

EXAMPLES

The present invention is further described in the following Examples, which illustrate reductions to practice of the invention but do not completely define all of its possible variations.

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, Hoboken, NJ (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), New England Biolabs, Inc. (Beverly, MA), 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. Pat. 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. Unless otherwise indicated herein 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” 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 is 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 strain ATCC #20362 was purchased from the American Type Culture Collection (Rockville, MD). *Yarrowia lipolytica* strains were routinely 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 + Uracil (MM+uracil or MMU) (per liter): Prepare MM media as above and add 0.1 g uracil and 0.1 g uridine.

Minimal Media + Uracil + Sulfonylurea (MMU+SU) (per liter): Prepare MMU media as above and add 280 mg sulfonylurea.

Minimal Media + Leucine + Lysine (MMLeuLys) (per liter): Prepare MM media as above and add 0.1 g leucine and 0.1 g lysine.

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).

High Glucose Media (HGM) (per liter): 80 glucose, 2.58 g KH_2PO_4 and 5.36 g K_2HPO_4 , pH 7.5 (do not need to adjust).

Fermentation medium without Yeast Extract (FM without YE) (per liter): 6.70

g Yeast Nitrogen base, 6.00 g KH_2PO_4 , 2.00 g K_2HPO_4 , 1.50 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ and 20 g Glucose.

- 5 Fermentation medium (FM) (per liter): Prepare FM without YE media as above and add 5.00 g Yeast extract (BBL).

Synthetic Dextrose Media (SD) (per liter): 6.7 g Yeast Nitrogen base with ammonium sulfate and without amino acids; and 20 g glucose.

10

Complete Minimal Glucose Broth Minus Uracil (CSM-Ura): Catalog No. C8140, Teknova, Hollister, CA (0.13% amino acid dropout powder minus uracil. 0.17% yeast nitrogen base, 0.5% $(\text{NH}_4)_2\text{SO}_4$, 2.0% glucose).

15

Transformation of *Y. 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 hr. Several large loopfuls of cells were scraped from the plate and resuspended in 1 mL of transformation buffer containing: 2.25 mL of 50% PEG, average MW 3350; 0.125 mL of 2 M Li acetate, pH 6.0; and 0.125 mL of 2 M DTT. Then, approximately 500 ng of linearized plasmid DNA was incubated in 100 µl of resuspended cells, and maintained at 39 °C for 1 hr 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.

20

25

Fatty Acid Analysis Of *Yarrowia lipolytica*

30

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 ["FAMES"] 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.

5 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
10 spun. The upper layer was removed and analyzed by GC as described above.

EXAMPLE 1

Generation Of *Yarrowia lipolytica* Strain Y4086 To Produce About 14% EPA Of Total Lipids Via The $\Delta 9$ Elongase/ $\Delta 8$ Desaturase Pathway

15 The present Example describes the construction of strain Y4086, derived from *Yarrowia lipolytica* ATCC #20362, capable of producing about 14% EPA relative to the total lipids via expression of a $\Delta 9$ elongase/ $\Delta 8$ desaturase pathway (FIG. 3A).

The development of strain Y4086 required the construction of strain
20 Y2224 (a FOA resistant mutant from an autonomous mutation of the *Ura3* gene of wildtype *Yarrowia* strain ATCC #20362), strain Y4001 (producing 17% EPA with a *Leu*- phenotype), strain Y4001U (*Leu*- and *Ura*- phenotype), strain Y4036 (producing 18% DGLA with a *Leu*- phenotype), strain Y4036U (*Leu*- and *Ura*- phenotype) and strain Y4070 (producing 12% ARA with a *Ura*-
25 phenotype). Further details regarding the construction of strains Y2224, Y4001, Y4001U, Y4036, Y4036U and Y4070 are described in Example 7 of Int'l. App. Pub. No. WO 2008/073367.

The final genotype of strain Y4070 with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was *Ura3*-, *unknown 1*-, *unknown 3*-, *Leu*+, *Lys*+,
30 *GPD*::*FmD12*::*Pex20*, *YAT1*::*FmD12*::*OCT*, *YAT1*::*ME3S*::*Pex16*,

GPAT::EgD9e::Lip2, EXP1::EgD9eS::Lip1, FBAINm::EgD9eS::Lip2, FBAINm::EgD8M::Pex20, EXP1::EgD8M::Pex16, FBAIN::EgD5::Aco, EXP1::EgD5S::Pex20, YAT1::RD5S::OCT (wherein FmD12 is a *Fusarium moniliforme* Δ 12 desaturase gene [Int'l. App. Pub. No. WO 2005/047485];

5 ME3S is a codon-optimized C_{16/18} elongase gene, derived from *Mortierella alpina* [Int'l. App. Pub. No. WO 2007/046817]; EgD9e is a *Euglena gracilis* Δ 9 elongase gene [Int'l. App. Pub. No. WO 2007/061742]; EgD9eS is a codon-optimized Δ 9 elongase gene, derived from *Euglena gracilis* [Int'l. App. Pub. No. WO 2007/061742]; EgD8M is a synthetic mutant Δ 8 desaturase [Int'l. App. Pub. No. WO 2008/073271], derived from *Euglena gracilis* [U.S. Pat. No. 7,256,033]; EgD5 is a *Euglena gracilis* Δ 5 desaturase [U.S. Pat. App. Pub. US 2007-0292924-A1]; EgD5S is a codon-optimized Δ 5 desaturase gene, derived from *Euglena gracilis* [U.S. Pat. App. Pub. No. 2007-0292924] and RD5S is a codon-optimized Δ 5 desaturase, derived from *Peridinium sp.*

10 CCMP626 [U.S. Pat. App. Pub. No. 2007-0271632]).

Generation Of Y4086 Strain To Produce About 14% EPA Of Total Lipids

Construct pZP3-Pa777U (FIG. 3B; SEQ ID NO:28), described in Table 19 of Int'l. App. Pub. No. WO 2008/07336 was generated to integrate three Δ 17 desaturase genes into the *Pox3* loci (GenBank Accession No. AJ001301

20 of strain Y4070, to thereby enable production of EPA. The Δ 17 desaturase genes were PaD17, a *Pythium aphanidermatum* Δ 17 desaturase (Int'l. App. Pub. No. WO 2008/054565), and PaD17S, a codon-optimized Δ 17 desaturase derived from *Pythium aphanidermatum* (Int'l. App. Pub. No. WO 2008/054565).

25 The pZP3-Pa777U plasmid was digested with *AscI*/*SphI*, and then used for transformation of strain Y4070 according to the General Methods. The transformant cells were plated onto MM plates and maintained at 30 °C for 2 to 3 days. Single colonies were re-streaked onto MM plates, and then inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2

30 days. The cells were collected by centrifugation, lipids were extracted, and

FAMES were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of EPA in the transformants containing the 3 chimeric genes of pZP3-Pa777U, but not in the parent Y407
 5 strain. Most of the selected 96 strains produced 10-13% EPA of total lipids. There were 2 strains (i.e., #58 and #79) that produced about 14.2% and 13.8% EPA of total lipids. These two strains were designated as Y4085 and Y4086, respectively.

The final genotype of strain Y4086 with respect to wildtype *Yarrowia*
 10 *lipolytica* ATCC #20362 was *Ura3+*, *Leu+*, *Lys+*, *unknown 1-*, *unknown 2-*, *YALI0F24167g-*, *GPD::FmD12::Pex20*, *YAT1::FmD12::OCT*, *YAT1::ME3S::Pex16*, *GPAT::EgD9e::Lip2*, *EXP1::EgD9eS::Lip1*, *FBAINm::EgD9eS::Lip2*, *FBAINm::EgD8M::Pex20*, *EXP1::EgD8M::Pex16*, *FBAIN::EgD5::Aco*, *EXP1::EgD5S::Pex20*, *YAT1::RD5S::OCT*,
 15 *YAT1::PaD17S::Lip1*, *EXP1::PaD17::Pex16*, *FBAINm::PaD17::Aco*.

EXAMPLE 2

Generation Of *Yarrowia lipolytica* Strain Y4128 To Produce About 37% EPA Of Total Lipids Via The $\Delta 9$ Elongase/ $\Delta 8$ Desaturase Pathway

The present Example describes the construction of strain Y4128,
 20 derived from *Yarrowia lipolytica* ATCC #20362, capable of producing about 37.6% EPA relative to the total lipids (i.e., greater than a 2-fold increase in EPA concentration as percent of total fatty acids with respect to Y4086; FIG. 3A).

The development of strain Y4128 required the construction of strains
 25 Y2224, Y4001, Y4001U, Y4036, Y4036U, Y4070 and Y4086 (described in Example 1), as well as construction of strain Y4086U1 (*Ura-*).

Generation Of Strain Y4086U1 (*Ura-*)

Strain Y4086U1 was created via temporary expression of the *Cre* recombinase enzyme in construct pY117 (FIG. 4A; SEQ ID NO:29; described
 30 in Table 20 of Int'l. App. Pub. No. WO 2008/073367) within strain Y4086 to

produce a *Ura*- phenotype. This released the LoxP sandwiched *Ura3* gene from the genome. The mutated *Yarrowia* acetohydroxyacid synthase ["AHAS"; E.C. 4.1.3.18] enzyme (i.e., GenBank Accession No. XP_501277, comprising a W497L mutation as set forth in SEQ ID NO:27; see Int'l. App. Pub. No. WO 2006/052870) in plasmid pY117 conferred sulfonyl urea herbicide resistance (SU^R), which was used as a positive screening marker.

Plasmid pY117 was used to transform strain Y4086 according to the General Methods. Following transformation, the cells were plated onto MMU+SU (280 µg/mL sulfonylurea; also known as chlorimuron ethyl, E. I. duPont de Nemours & Co., Inc., Wilmington, DE) plates and maintained at 30 °C for 2 to 3 days. The individual SU^R colonies grown on MMU+SU plates were picked, and streaked into YPD liquid media at 30 °C and shaken at 250 rpm/min for 1 day to cure the pY117 plasmid. The grown cultures were streaked onto MMU plates. After two days at 30 °C, the individual colonies were re-streaked onto MM and MMU plates. Those colonies that could grow on MMU, but not on MM plates were selected. Two of these strains with *Ura* phenotypes were designated as Y4086U1 and Y4086U2.

Generation Of Y4128 Strain To Produce About 37% EPA Of Total Lipids

Construct pZP2-2988 (FIG. 4B; SEQ ID NO:30; described in Table 21 of Int'l. App. Pub. No. WO 2008/073367) was generated to integrate one $\Delta 12$ desaturase gene (i.e., FmD12S, a codon-optimized $\Delta 12$ desaturase gene derived from *Fusarium moniliforme* [Int'l. App. Pub. No. WO 2005/047485]), two $\Delta 8$ desaturase genes (i.e., EgD8M) and one $\Delta 9$ elongase gene (i.e., EgD9eS) into the *Pox2* loci (GenBank Accession No. AJ001300) of strain Y4086U1, to thereby enable higher level production of EPA. The pZP2-2988 plasmid was digested with *Ascl*/*Sph*I, and then used for transformation of strain Y4086U1 according to the General Methods. The transformant cells were plated onto MM plates and maintained at 30 °C for 2 to 3 days. Single colonies were re-streaked onto MM plates, and then inoculated into liquid MMLeuLys at 30 °C and shaken at 250 rpm/min for 2

days. The cells were collected by centrifugation, resuspended in HGM and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, lipids were extracted, and FAMES were prepared by transesterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

5 GC analyses showed that most of the selected 96 strains produced 12 15.6% EPA of total lipids. There were 2 strains (i.e., #37 within Group I and #33 within Group II) that produced about 37.6% and 16.3% EPA of total lipids. These two strains were designated as Y4128 and Y4129, respectively

The final genotype of strain Y4128 with respect to wildtype *Yarrowia*
 10 *lipolytica* ATCC #20362 was: *YALI0F24167g*-, *Pex10*-, *unknown 1*-, *unknownr*
2-, *GPD::FmD12::Pex20*, *YAT1::FmD12::OCT*, *GPM/FBAIN::FmD12S::OCT*,
YAT1::ME3S::Pex16, *GPAT::EgD9e::Lip2*, *EXP1::EgD9eS::Lip1*,
FBAINm::EgD9eS::Lip2, *FBA::EgD9eS::Pex20*, *FBAINm::EgD8M::Pex20*,
EXP1::EgD8M::Pex16, *GPDIN::EgD8M::Lip1*, *YAT1::EgD8M::Aco*,
 15 *FBAIN::EgD5::Aco*, *EXP1::EgD5S::Pex20*, *YAT1::RD5S::OCT*,
YAT1::PaD17S::Lip1, *EXP1::PaD17::Pex16*, *FBAINm::PaD17::Aco*.

Yarrowia lipolytica strain Y4128 was deposited with the American Type Culture Collection on August 23, 2007 and bears the designation ATCC PTA 8614.

20 Generation Of Y4128U Strains With A Ura- Phenotype

In order to disrupt the *Ura3* gene in strain Y4128, construct pZKUE3S (FIG. 5A; SEQ ID NO:31; described in Table 22 of Int'l. App. Pub. No. WO 2008/073367) was created to integrate a *EXP1::ME3S::Pex20* chimeric gene into the *Ura3* gene of strain Y4128. Plasmid pZKUE3S was digested with
 25 *SphI/PacI*, and then used to transform strain Y4128 according to the General Methods. Following transformation, cells were plated onto MM + 5-FOA selection plates and maintained at 30 °C for 2 to 3 days.

A total of 24 transformants grown on MM + 5-FOA selection plates were picked and re-streaked onto fresh MM + 5-FOA plates. The cells were
 30 stripped from the plates, lipids were extracted, and FAMES were prepared by

trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of between 10-15% EPA in all of the transformants with pZKUE3S from plates. The strains identified as #3, #4, #10, #12, #19 and #21 that produced 12.9%, 14.4%, 15.2%, 15.4%, 14% and 10.9% EPA of total lipids were designated as Y4128U1, Y4128U2, Y4128U3, Y4128U4, Y4128U5 and Y4128U6, respectively (collectively, Y4128U).

The discrepancy in the % EPA quantified in Y4128 (37.6%) versus Y4128U (average 13.8%) is based on differing growth conditions. Specifically, the former culture was analyzed following two days of growth in liquid culture, while the latter culture was analyzed after growth on an agar plate. The Applicants have observed a 2-3 fold increase in % EPA, when comparing results from agar plates to those in liquid culture. Thus, although results are not directly comparable, both Y4128 and Y4128U strains demonstrate production of EPA.

EXAMPLE 3

Determination Of Total Lipid Content Of *Yarrowia lipolytica* Strain Y4128

The total amount of lipid produced by strain Y4128 and the percentage of each fatty acid species in the lipid were measured by GC analysis. Specifically, total lipids were extracted, and FAMES were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC, as described in the General Methods.

Dry cell weight was determined by collecting cells from 10 mL of culture via centrifugation, washing the cells with water once to remove residual medium, drying the cells in a vacuum oven at 80 °C overnight, and weighing the dried cells. The total amount of FAMES in a sample was determined by comparing the areas of all peaks in the GC profile with the peak area of an added known amount of internal standard C15:0 fatty acid.

Based on the above analyses, lipid content as a percentage of dry cell weight (DCW) and lipid composition was determined for strains Y4086 and Y4128. Strain Y4128 had decreased lipid content with respect to strain Y4086 (11.2 TFAs % DCW versus 28.6 TFAs % DCW). In contrast, strain Y4128 had elevated EPA concentrations among lipids with respect to strain Y4086, as shown below in Table 6. Fatty acids are identified as 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA, DGLA, ETrA, ETA and EPA; fatty acid compositions were expressed as the weight percent (wt. %) of total fatty acid (TFAs).

Table 6

Lipid Composition In *Yarrowia lipolytica* Strains Y4086 And Y4128

Sample	18:0	18:1	18:2 [LA]	18:3 (n-3) [ALA]	20:2 [EDA]	20:3 (n-6) [DGLA]	20:3 (n-3) [ETrA]	20:4 (n-3) [ETA]	20:5 (n-3) [EPA]
Y4086	4.6	26.8	28.0	6.9	7.6	0.9	4.9	2.0	9.8
Y4128	1.8	6.7	19.6	1.8	4.2	3.4	1.5	6.0	42.8

EPA content in the cell, expressed as mg EPA/g dry cell and calculated according to the following formula: (% of EPA/Lipid) * (% of Lipid /dry cell weight) * 0.1, increased from 28 mg EPA/g DCW in strain Y4086 to 47.9 mg EPA/g DCW in strain Y4128.

Thus, the results in Table 6 showed that compared to the parent strain Y4086, strain Y4128 had a lower total lipid content (TFAs % DCW) (11.2% versus 28.6%), higher EPA % TFAs (42.8% versus 9.8%), and higher **EPA % DCW (4.8% versus 2.8%)**. Additionally, strain Y4128 had a 3.3-fold increase in the amount of EPA relative to the total PUFAs (54% of the PUFAs [as a % TFAs] versus 16.3% of the PUFAs [as a % TFAs]) and a 1.7-fold increase in the amount of C20 PUFAs relative to the total PUFAs (73% of the PUFAs [as a % TFAs] versus 42% of the PUFAs [as a % TFAs]).

EXAMPLE 4

Determination Of The Integration Site Of pZP2-2988 In *Yarrowia lipolytica*
Strain Y4128 As A Pex10 Integration

The genomic integration site of pZP2-2988 in strain Y4128 was determined by genome walking using the Universal GenomeWalker™ Kit from Clontech (Palo Alto, CA), following the manufacturer's recommended protocol. Based on the sequence of the plasmid, the following primers were designed for genome walking: pZP-GW-5-1 (SEQ ID NO:32), pZP-GW-5-2 (SEQ ID NO:33), pZP-GW-5-3 (SEQ ID NO:34), pZP-GW-5-4 (SEQ ID NO:35), pZP-GW-3-1 (SEQ ID NO:36), pZP-GW-3-2 (SEQ ID NO:37), pZP-GW-3-3 (SEQ ID NO:38) and pZP-GW-3-4 (SEQ ID NO:39).

Genomic DNA was prepared from strain Y4128 using the Qiagen Miniprep kit with a modified protocol. Cells were scraped off a YPD medium plate into a 1.5 mL microfuge tube. Cell pellet (100 µl) was resuspended with 250 µl of buffer P1 containing 0.125 M β-mercaptoethanol and 1 mg/mL zymolyase 20T (MP Biomedicals, Inc., Solon, OH). The cell suspension was incubated at 37 °C for 30 min. Buffer P2 (250 µl) was then added to the tube. After mixing by inverting the tube for several times, 350 µl of buffer N3 was added. The mixture was then centrifuged at 14,000 rpm for 5 min in a microfuge. Supernatant was poured into a Qiagen miniprep spin column, and centrifuged for 1 min. The column was washed once by adding 0.75 mL of buffer PE, followed by centrifugation at 14,000 rpm for 1 min. The column was dried by further centrifugation at 14,000 rpm for 1 min. Genomic DNA was eluted by adding 50 µl of buffer EB to the column, allowed to sit for 1 min and centrifuged at 14,000 rpm for 1 min.

Purified genomic DNA was used for genome walking. The DNA was digested with restriction enzymes *DraI*, *EcoRV*, *PvuII* and *StuI* separately, according to the protocol of the GenomeWalker kit. For each digestion, the reaction mixture contained 10 µl of 10X restriction buffer, 10 µl of the appropriate restriction enzyme and 8 µg of genomic DNA in a total volume of 100 µl. The reaction mixtures were incubated at 37 °C for 4 hrs. The

digested DNA samples were then purified using Qiagen PCR purification kit following the manufacturer's protocol exactly. DNA samples were eluted in 16 µl water. Purified, digested genomic DNA samples were then ligated to the genome walker adaptor (*infra*). Each ligation mixture contained 1.9 µl of the genome walker adaptor, 1.6 µl of 10X ligation buffer, 0.5 µl T4 DNA ligase and 4 µl of the digested DNA. The reaction mixtures were incubated at 16 °C overnight. Then, 72 µl of 50 mM TrisHCl, 1 mM EDTA, pH 7.5 were added to each ligation mixture.

For 5'-end genome walking, four PCR reactions were carried out using 1 µl of each ligation mixture individually as template. In addition, each reaction mixture contained 1 µl of 10 µM primer pZP-GW-5-1 (SEQ ID NO:32), 1 µl of 10 µM kit-supplied Genome Walker adaptor, 41 µl water, 5 µl 10X cDNA PCR reaction buffer and 1 µl Advantage cDNA polymerase mix from Clontech. The sequence of the Genome Walker adaptor (SEQ ID NOs:40 [top strand] and 41 [bottom strand]), is shown below:

5' -GTAATACGACTCACTATAGGGCACGCGTGGTCGACGGCCCGGGCTGGT-3'
3' -H2N-CCCGACCA-5'

The PCR conditions were as follows: 95 °C for 1 min, followed by 30 cycles at 95 °C for 20 sec and 68 °C for 3 min, followed by a final extension at 68 °C for 7 min. The PCR products were each diluted 1:100 and 1 µl of the diluted PCR product used as template for a second round of PCR. The conditions were exactly the same except that pZP-GW-5-2 (SEQ ID NO:33) replaced pZP-GW-5-1 (SEQ ID NO:32).

For 3'-end genome walking, four PCR reactions were carried out as above, except primer pZP-GW-3-1 (SEQ ID NO:36) and nested adaptor primer (SEQ ID NO:42) were used. The PCR products were similarly diluted and used as template for a second round of PCR, using pZP-GW-3-2 (SEQ ID NO:37) to replace pZP-GW-3-1 (SEQ ID NO:36).

PCR products were analyzed by gel electrophoresis. One reaction product, using *EcoRV* digested genomic DNA as template and the primers pZP-GW-3-2 and nested adaptor primer, generated a ~1.6 kB fragment. This fragment was isolated, purified with a Qiagen gel purification kit and cloned
 5 into pCR2.1-TOPO. Sequence analysis showed that the fragment included both part of plasmid pZP2-2988 and the *Yarrowia* genomic DNA from chromosome C. The junction between them was at nucleotide position 139826 of chromosome C. This was inside the coding region of the Pex10 gene (GenBank Accession No. CAG81606; SEQ ID NO:10).

10 To determine the 5' end of the junction, PCR amplification was performed using genomic DNA from strain Y4128 as the template and primers Per10 F1 (SEQ ID NO:43) and ZPGW-5-5 (SEQ ID NO:44). The reaction mixture included 1 µl each of 20 µM primer, 1 µl genomic DNA, 22 µl water and 25 µl TaKaRa ExTaq 2X premix (TaKaRa Bio Inc., Otsu Shiga,
 15 Japan). The thermocycler conditions were: 94 °C for 1 min, followed by 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec and 72 °C for 2 min, followed by final extension at 72 °C for 7 min. A 1.6 kB DNA fragment was amplified and cloned into pCR2.1-TOPO. Sequence analysis showed that it was a chimeric fragment between *Yarrowia* genomic DNA from chromosome C and pZP2-
 20 2988. The junction was at nucleotide position 139817 of chromosome C. Thus, a 10 nucleotide segment of chromosome C was replaced by the *Ascl/SphI* fragment from pZP2-2988 (FIG. 4B) in strain Y4128. As a result, Pex10 in strain Y4128 was lacking the last 32 amino acids of the encoded protein.

25 Based on the above conclusions, the Y4128U strains isolated in Example 2 (*supra*) are referred to subsequently as Δpex10 strains. For clarity, strain Y4128U1 is equivalent to strain Y4128U1 (Δpex10).

EXAMPLE 5

Plasmid Expression Of Pex10 In *Yarrowia lipolytica* Strain Y4128U1 (Δpex10)

Three plasmids that carried the *Y. lipolytica* Pex10 gene were constructed: 1) pFBAIn-PEX10 allowed the expression of the Pex10 ORF under the control of the FBAInm promoter; and, 2) pPEX10-1 and pPEX10-2 allowed the expression of Pex10 under control of the native Pex10 promoter, although pPEX10-1 used a shorter version (~500 bp) while pPEX10-2 used a longer version (~900 bp) of the promoter. Following construction of these expression plasmids and transformation, the effect of Pex10 plasmid expression on total oil and on EPA level in the *Y. lipolytica* strain Y4128U1 (Δ pex10) was determined. Deletion of Pex10 resulted in an increased amount of EPA as a percent of TFAs, but a reduced amount of total lipid, as percent of DCW, in the cell.

Construction Of pFBAIn-PEX10, pPEX10-1 And pPEX10-2

To construct pFBAIn-PEX10, the primers Per10 F1 (SEQ ID NO:43) and Per10 R (SEQ ID NO:45) were used to amplify the coding region of the Pex10 gene using *Y. lipolytica* genomic DNA as template. The PCR reaction mixture contained 1 μ l each of 20 μ M primers, 1 μ l of *Y. lipolytica* genomic DNA (~100 ng), 25 μ l ExTaq 2X premix and 22 μ l water. The reaction was carried out as follows: 94 °C for 1 min, followed by 30 cycles of 94 °C for 20 sec, 55 °C for 20 sec and 72 °C for 90 sec, followed by a final extension of 72 °C for 7 min. The PCR product, a 1168 bp DNA fragment, was purified with a Qiagen PCR purification kit, digested with *Nco*I and *Not*I, and cloned into pFBAIn-MOD-1 (SEQ ID NO:46; FIG. 5B) digested with the same two restriction enzymes.

Of the 8 individual clones subjected to sequence analysis, 2 had the correct sequence of Pex10 with no errors. The components of pFBAIn-PEX10 (SEQ ID NO:47; FIG. 6A) are listed below in Table 7.

Table 7

Components Of Plasmid pFBAIn-PEX10 (SEQ ID NO:47)

RE Sites And Nucleotides	Description Of Fragment And Chimeric Gene Components
--------------------------	--

Within SEQ ID NO:47	
<i>Bgl</i> II- <i>Bs</i> IWI (6040-318)	FBAINm::Pex10::Pex20, comprising: • FBAINm: <i>Yarrowia lipolytica</i> FBAINm promoter (U.S. Pat. No. 7,202,356); • Pex10: <i>Y. lipolytica</i> Pex10 ORF (GenBank Accession No. AB036770, nucleotides 1038-2171; SEQ ID NO:21); • Pex20: Pex20 terminator sequence from <i>Yarrowia</i> Pex20 gene (GenBank Accession No. AF054613)
<i>Pac</i> I- <i>Bgl</i> II (4530-6040)	<i>Yarrowia URA3</i> (GenBank Accession No. AJ306421)
(3123-4487)	<i>Yarrowia</i> autonomous replicating sequence 18 (ARS18; GenBank Accession No. A17608)
(2464-2864)	<i>E. coli</i> f1 origin of replication
(1424-2284)	Ampicillin-resistance gene (Amp ^R) for selection in <i>E. coli</i>
(474-1354)	<i>ColE1</i> plasmid origin of replication

To construct pPEX10-1 and pPEX10-2, primers PEX10-R-*Bs*iWI (SEQ ID NO:48), PEX10-F1-*Sa*II (SEQ ID NO:49) and PEX10-F2-*Sa*II (SEQ ID NO:50) were designed and synthesized. PCR amplification using genomic

5 *Yarrowia lipolytica* DNA and primers PEX10-R-*Bs*iWI and PEX10-F1-*Sa*II generated a 1873 bp fragment containing the Pex10 ORF, 500 bp of the 5' upstream region and 215 bp of the 3' downstream region of the Pex10 gene, flanked by *Sa*II and *Bs*iWI restriction sites at either end. This fragment was purified with the Qiagen PCR purification kit, digested with *Sa*II and *Bs*iWI,

10 and cloned into pEXP-MOD-1 (SEQ ID NO:51; FIG. 6B) digested with the same two enzymes to generate pPEX10-1 (SEQ ID NO:52; FIG. 7A). Plasmid pEXP-MOD1 is similar to pFBAIN-MOD-1 (SEQ ID NO:46; FIG. 5B) except that the FBAINm promoter in the latter was replaced with the EXP1 promoter. Table 8 lists the components of pPEX10-1.

15

Table 8Components Of Plasmid pPEX10-1 (SEQ ID NO:52)

RE Sites And Nucleotides Within SEQ ID NO:52	Description Of Fragment And Chimeric Gene Components
--	--

<i>Sall</i> - <i>Bsi</i> WI (5705-1)	Pex10-5':Pex10::Pex10-3', comprising: • Pex10-5': 500 bp of the 5' promoter region of <i>Yarrowia lipolytica</i> Pex10 gene; • Pex10: <i>Yarrowia lipolytica</i> Pex10 ORF (GenBank Accession No. AB036770, nucleotides 1038-2171; SEQ ID NO:21); • Pex10-3': 215 bp of Pex10 terminator sequence from <i>Yarrowia</i> Pex10 gene (GenBank Accession No. AB036770) [Note the entire Pex10-5':Pex10::Pex10-3' expression cassette is labeled collectively as "PEX10" in the Figure]
<i>PacI</i> - <i>Sall</i> (4216-5703)	<i>Yarrowia URA3</i> gene (GenBank Accession No. AJ306421)
(2806-4170)	<i>Yarrowia</i> autonomous replicating sequence 18 (ARS18; GenBank Accession No. A17608)
(2147-2547)	<i>E. coli</i> f1 origin of replication
(1107-1967)	Ampicillin-resistance gene (Amp ^R) for selection in <i>E. coli</i>
(157-1037)	<i>ColE1</i> plasmid origin of replication

PCR amplification of *Yarrowia lipolytica* genomic DNA using PEX10-R-*Bsi*WI (SEQ ID NO:48) and PEX10-F2-*Sall* (SEQ ID NO:50) generated a 2365 bp fragment containing the PEX10 ORF, 991 bp of the 5' upstream region and 215 bp of the 3' downstream region of the Pex10 gene, flanked by *Sall* and *Bsi*WI restriction sites at either end. This fragment was purified with a Qiagen PCR purification kit, digested with *Sall* and *Bsi*WI, and cloned into similarly digested pEXP-MOD-1. This resulted in synthesis of pPEX10-2 (SEQ ID NO:53), whose construction is analogous to that of plasmid pPEX10-1 (Table 8, *supra*), with the exception of the longer Pex10-5' promoter in the chimeric Pex10-5':Pex10::Pex10-3' gene.

Expression Of Pex10 In Strain Y4128U1 (Δ pex10)

Plasmids pFBAIN-MOD-1 (control; SEQ ID NO:46), pFBAln-PEX10 (SEQ ID NO:47), pPEX10-1 (SEQ ID NO:52) and pPEX10-2 (SEQ ID NO:53) were transformed into Y4128U1 (Δ pex10) according to the protocol in the General Methods. Transformants were plated on MM plates. The total lipid content and fatty acid composition of transformants carrying the above plasmids were analyzed as described in Example 3.

Lipid content as a percentage of dry cell weight (DCW) and lipid composition are shown below in Table 9. Specifically, fatty acids are identified as 18:0 (stearic acid), 18:1 (oleic acid), LA, ALA, EDA, DGLA, ETrA, ETA and EPA; fatty acid compositions were expressed as the weight percent (wt. %) of total fatty acids.

Table 9

Lipid Composition In *Yarrowia lipolytica* Strain Y4128U1 (Δ pex10) Transformed With Various Pex10 Plasmids

Plasmid	TFA % DCW	18:0	18:1	18:2 [LA]	18:3 (ω 3) [ALA]	20:2 [EDA]	20:3 (ω 6) [DGLA]	20: 3 (ω 3) [ETrA]	20:4 (ω 3) [ETA]	20:5 (ω 3) [EPA]
pFBAIN-MOD-1	22.8	1.9	9.6	18.3	2.0	4.3	2.3	2.1	5.9	27.7
pFBAIN-PEX10	29.2	4.0	24.9	25.1	7.6	6.6	1.0	5.3	3.6	10.8
pPEX10-1	27.1	3.9	25.0	25.2	8.2	6.4	0.9	5.2	3.5	10.7
pPEX10-2	28.5	4.3	25.4	24.5	7.6	6.4	1.0	5.3	3.4	10.8

The results in Table 9 showed that expression of Pex10 in Y4128U1 (Δ pex10), either from the native *Y. lipolytica* Pex10 promoter or from the *Y. lipolytica* FBAlNm promoter, reduced the percent of EPA back to the level of Y4086 while increasing the total lipid content (TFA % DCW) up to the level of Y4086 (see data of Table 6 for comparison). EPA content per gram of dry cell changed from 63.2 mg in the case of the control sample (i.e., cells carrying pFBAln-MOD-1) to 31.5 mg in cells carrying pFBAln-PEX10, 29 mg in cells carrying pPEX10-1 and 30.8 mg in cells carrying pPEX10-2. These results demonstrated that disruption of the ring-finger domain of Pex10 increased the amount of EPA but reduced the **total lipid content in the cell**.

Thus, the results in Table 9 showed that compared to Y4128U1 (Δ pex10) transformant with control plasmid, all transformants with Pex10 expressing plasmids showed higher lipid content (TFAs % DCW) (>27% versus 22.8%), lower EPA % TFAs (ca. 10.8% versus 27.7%), and lower EPA % DCW (<3.1% versus 6.3%). Additionally, strain Y4128U1 (Δ pex10) transformant with control plasmid, as compared to those transformants with Pex10 expressing plasmids, had a 2.5-fold increase in the amount of EPA relative to the total PUFAs (44% of the PUFAs [as a % TFAs] versus 17.5% (avg) of the PUFAs [as a % TFAs]) and a 1.5-fold increase in the amount of C20 PUFAs relative to the total PUFAs (67% of the PUFAs [as a % TFAs] versus 44% (avg) of the PUFAs [as a % TFAs]).

EXAMPLE 6

Generation Of Y4184U Strain To Produce EPA

Y. lipolytica strain Y4184U was used as the host in Example 7, *infra*. Strain Y4184U was derived from *Y. lipolytica* ATCC #20362, and is capable of producing EPA via expression of a Δ 9 elongase/ Δ 8 desaturase pathway. The strain has a *Ura*- phenotype and its construction is described in Example 7 of Int'l. App. Pub. No. WO 2008/073367.

In summary, however, the development of strain Y4184U required the construction of strain Y2224, strain Y4001, strain Y4001U, strain Y4036,

strain Y4036U and strain Y4069 (*supra*, Example 1). Further development of strain Y4184U (diagrammed in Figure 7B) required generation of strain Y4084, strain Y4084U1, strain Y4127 (deposited with the American Type Culture Collection on November 29, 2007, under accession number ATCC
 5 PTA-8802), strain Y4127U2, strain Y4158, strain Y4158U1 and strain Y4184. The plasmid construct pZKL1-2SP98C, used for transformation of strain Y4127U2, is diagrammed in FIG. 8A (SEQ ID NO:54; described in Table 23 of Int'l. App. Pub. No. WO 2008/073367). Plasmid pZKL2-5U89GC, used for transformation of strain Y4158U1, is shown in FIG. 8B (SEQ ID NO:55;
 10 described in Table 24 of Int'l. App. Pub. No. WO 2008/073367).

The final genotype of strain Y4184 (producing 31% EPA of total lipids) with respect to wildtype *Yarrowia lipolytica* ATCC #20362 was *unknown 1-*, *unknown 2-*, *unknown 4-*, *unknown 5-*, *unknown 6-*, *unknown 7-*, YAT1::ME3S::Pex16, EXP1::ME3S::Pex20 (2 copies), GPAT::EgD9e::Lip2, FBAINm::EgD9eS::Lip2, EXP1::EgD9eS::Lip1, FBA::EgD9eS::Pex20,
 15 YAT1::EgD9eS::Lip2, GPD::EgD9eS::Lip2, GPDIN::EgD8M::Lip1, YAT1::EgD8M::Aco, EXP1::EgD8M::Pex16, FBAINm::EgD8M::Pex20, FBAIN::EgD8M::Lip1 (2 copies), GPM/FBAIN::FmD12S::Oct, EXP1::FmD12S::Aco, YAT1::FmD12::Oct, GPD::FmD12::Pex20,
 20 EXP1::EgD5S::Pex20, YAT1::EgD5S::Aco, YAT1::Rd5S::Oct, FBAIN::EgD5::Aco, FBAINm::PaD17::Aco, EXP1::PaD17::Pex16, YAT1::PaD17S::Lip1, YAT1::YICPT1::Aco, GPD::YICPT1::Aco (wherein FmD12 is a *Fusarium moniliforme* $\Delta 12$ desaturase gene [Int'l. App. Pub. No. WO 2005/047485]; FmD12S is a codon-optimized $\Delta 12$ desaturase gene,
 25 derived from *Fusarium moniliforme* [Int'l. App. Pub. No. WO 2005/047485]; ME3S is a codon-optimized C_{16/18} elongase gene, derived from *Mortierella alpina* [Int'l. App. Pub. No. WO 2007/046817]; EgD9e is a *Euglena gracilis* $\Delta 9$ elongase gene [Int'l. App. Pub. No. WO 2007/061742]; EgD9eS is a codon-optimized $\Delta 9$ elongase gene, derived from *Euglena gracilis* [Int'l. App. Pub.
 30 No. WO 2007/061742]; EgD8M is a synthetic mutant $\Delta 8$ desaturase [Int'l.

App. Pub. No. WO 2008/073271], derived from *Euglena gracilis* [U.S. Pat. No. 7,256,033]; EgD5 is a *Euglena gracilis* $\Delta 5$ desaturase [U.S. Pat. App. Pub. US 2007-0292924-A1]; EgD5S is a codon-optimized $\Delta 5$ desaturase gene, derived from *Euglena gracilis* [U.S. Pat. App. Pub. No. 2007-0292924]
 5 RD5S is a codon-optimized $\Delta 5$ desaturase, derived from *Peridinium sp.* CCMP626 [U.S. Pat. App. Pub. No. 2007-0271632]; PaD17 is a *Pythium aphanidermatum* $\Delta 17$ desaturase [Int'l. App. Pub. No. WO 2008/054565]; PaD17S is a codon-optimized $\Delta 17$ desaturase, derived from *Pythium aphanidermatum* [Int'l. App. Pub. No. WO 2008/054565]; and, YICPT1 is a
 10 *Yarrowia lipolytica* diacylglycerol cholinephosphotransferase gene [Int'l. App. Pub. No. WO 2006/052870]).

In order to disrupt the *Ura3* gene in strain Y4184, construct pZKUE3S (FIG. 5A; SEQ ID NO:31; described in Table 22 of Int'l. App. Pub. No. WO 2008/073367) was used to integrate a EXP1::ME3S::Pex20 chimeric gene
 15 into the *Ura3* gene of strain Y4184 to result in strains Y4184U1 (11.2% EPA of total lipids), Y4184U2 (10.6% EPA of total lipids) and Y4184U4 (15.5% EPA of total lipids), respectively (collectively, Y4184U).

EXAMPLE 7

Chromosomal Deletion Of Pex10 In *Yarrowia lipolytica* Strain Y4184U4

Increases Accumulation Of EPA And Total Lipid Content

Construct pYPS161 (FIG. 9A, SEQ ID NO:56) was used to knock out the chromosomal Pex10 gene from the EPA-producing *Yarrowia* strain Y4184U4 (Example 6). Transformation of *Y. lipolytica* strain Y4184U4 with the Pex10 knock out construct resulted in creation of strain Y4184 ($\square$ pex10).
 25 The effect of the Pex10 knockout on total oil and on EPA level was determined and compared. Specifically, knockout of Pex10 resulted in an increased percentage of EPA (as % TFAs and % DCW) and an increased total lipid content in the cell.

Construct pYSP161

30 The construct pYPS161 contained the following components:

Table 10Description of Plasmid pYPS161 (SEQ ID NO:56)

RE Sites And Nucleotides Within SEQ ID NO:56	Description Of Fragment And Chimeric Gene Components
<i>Ascl/BsiWI</i> (1521-157)	1364 bp Pex10 knockout fragment #1 of <i>Yarrowia</i> Pex10 gene (GenBank Accession No. AB036770)
<i>Pacl/SphI</i> (5519-4229)	1290 bp Pex10 knockout fragment #2 of <i>Yarrowia</i> Pex10 gene (GenBank Accession No. AB036770)
<i>Sall/EcoRI</i> (7170-5551)	<i>Yarrowia URA3</i> gene (GenBank Accession No. AJ306421)
2451-1571	<i>ColE1</i> plasmid origin of replication
3369-2509	ampicillin-resistance gene (Amp ^R) for selection in <i>E. coli</i>
3977-3577	<i>E. coli</i> f1 origin of replication

Generation Of *Yarrowia lipolytica* Knockout Strain Y4184 (Δ Pex10)

5 Standard protocols were used to transform *Yarrowia lipolytica* strain Y4184U4 (Example 6) with the purified 5.3 kB *Ascl/SphI* fragment of Pex10 knockout construct pYPS161 (*supra*), and a cells alone control was also prepared. There were about 200 to 250 colonies present for each of the experimental transformations, while there were no colonies present on the
10 cells alone plates (per expectations).

 Colony PCR was used to screen for cells having the Pex10 deletion. Specifically, the PCR reaction was performed using MasterAmp Taq polymerase (Epicentre Technologies, Madison, WI) following standard protocols, using PCR primers Pex-10del1 3'.Forward (SEQ ID NO:57) and
15 Pex-10del2 5'.Reverse (SEQ ID NO:58). The PCR reaction conditions were 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 sec, 60 °C for 30 sec and 72 °C for 2 min, followed by a final extension at 72 °C for 6 min. The reaction was then held at 4 °C. If the Pex10 knockout construct integrated within the Pex10 region, a single PCR product 2.8 kB in size was expected to
20 be produced. In contrast, if the strain integrated the Pex10 knockout construct in a chromosomal region other than the Pex10 region, then two

PCR fragments, i.e., 2.8 kB and 1.1 kB, would be generated. Of the 288 colonies screened, the majority had the Pex10 knockout construct integrated at a random site. Only one of the 288 colonies contained the Pex10 knockout. This strain was designated Y4184 (Δ pex10).

5 Evaluation Of *Yarrowia lipolytica* Strains Y4184 And Y4184 (Δ Pex10) For Total Oil And EPA Production

To evaluate the effect of the Pex10 knockout on the percent of PUFAs in the total lipid fraction and the total lipid content in the cells, strains Y4184 and Y4184 (Δ pex10) were grown under comparable oleaginous conditions. Specifically, cultures were grown at a starting OD₆₀₀ of ~0.1 in 25 mL of either fermentation media (FM) or FM medium without Yeast Extract (FM without YE) in a 250 mL flask for 48 hrs. The cells were harvested by centrifugation for 10 min at 8000 rpm in a 50 mL conical tube. The supernatant was discarded and the cells were re-suspended in 25 mL of HGM and transferred to a new 250 mL flask. The cells were incubated with aeration for an additional 120 hrs at 30 °C.

To determine the dry cell weight (DCW), the cells from 5 mL of the FM grown cultures and 10 mL of the FM without YE-grown cultures were processed. The cultured cells were centrifuged for 10 min at 4300 rpm. The pellet was re-suspended using 10 mL of saline and was centrifuged under the same conditions for a second time. The pellet was then re-suspended using 1 mL of sterile H₂O (three times) and was transferred to a pre-weighed aluminum pan. The cells were dried overnight in a vacuum oven at 80 °C. The weight of the cells was determined.

25 The total lipid content and fatty acid composition of transformants carrying the above plasmids were analyzed as described in Example 3. DCW, total lipid content (TFAs % DCW), total EPA % TFAs, and EPA % DCW are shown below in Table 11.

Table 11

30 Lipid Composition In *Y. lipolytica* Strains Y4184 And Y4184 (Δ Pex10)

Media	Strain	DCW	TFAs % DCW	EPA % TFAs	EPA % DCW
FM	Y4184	11.5	11.8	20.6	2.4
	Y4184 (Δ Pex10)	11.5	17.6	43.2	7.6
FM without YE	Y4184	4.6	8.8	23.2	2.0
	Y4184 (Δ Pex10)	4.0	13.2	46.1	6.1

The results in Table 11 showed that knockout of the chromosomal Pex10 gene in Y4184 (Δ Pex10) increased the percent of EPA (as % TFAs and as % DCW) and increased the total oil content, as compared to the percent of EPA and total oil content in strain Y4184 whose native Pex10p has not been knocked out. More specifically, in FM media, there was about 109% increase in EPA (% TFAs), about 216% increase in EPA productivity (% DCW) and about 49% increase in total oil (TFAs % DCW). In FM without YE media, there was about 100% increase in EPA (% TFAs), about 205% increase in EPA productivity (% DCW) and about 50% increase in total oil (TFAs % DCW).

Thus, the results in Table 11 showed that in FM medium, compared to the parent strain Y4184, Y4184 (Δ Pex10) strain had higher lipid content (TFAs % DCW) (17.6% versus 11.8%), higher EPA % TFAs (43.2% versus 20.6%), and higher EPA % DCW (7.6% versus 2.4%). Similarly, in FM medium without YE, compared to the parent strain Y4184, Y4184 (Δ Pex10) strain had higher lipid content (TFAs % DCW) (13.2% versus 8.8%), higher EPA % TFAs (46.1% versus 23.2%), and higher EPA % DCW (6.1% versus 2.0%).

20 EXAMPLE 8 (Prophetic)

Chromosomal Knockout Of Alternate Pex Genes In PUFA-Producing Strains Of *Yarrowia lipolytica*

The present Example describes various strains of *Yarrowia lipolytica* that have been engineered to produce ω -3/ ω -6 PUFAs. It is contemplated that any of these *Y. lipolytica* host strains could be engineered to produce an increased amount of ω -3/ ω -6 PUFAs in the total lipid fraction and in the oil

fraction, if the chromosomal gene encoding Pex1p, Pex2p, Pex3p, Pex3Bp, Pex4p, Pex5p, Pex6p, Pex7p, Pex8p, Pex12p, Pex13p, Pex14p, Pex16p, Pex17p, Pex19p, Pex20p, Pex22p or Pex26p was disrupted using the methodology of Example 7, *supra*.

5 More specifically, a variety of *Yarrowia lipolytica* strains have been engineered by the Applicant's Assignee to produce high concentrations of various ω -3/ ω -6 PUFAs via expression of a heterologous Δ 6 desaturase/ Δ 6 elongase PUFA pathway or a heterologous Δ 9 elongase/ Δ 8 desaturase PUFA pathway.

10 Summary Of Representative *Yarrowia lipolytica* Strains Producing ω -3/ ω -6 PUFAs

Although some representative strains are summarized in the Table below, the disclosure of *Yarrowia lipolytica* strains producing ω -3/ ω -6 PUFAs is not limited in any way to the strains therein. Instead, all of the teachings
15 provided in the present Application, in addition to the following commonly owned and co-pending applications, are useful for development of a suitable *Yarrowia lipolytica* strain engineered to produce ω -3/ ω -6 PUFAs. These specifically include the following Applicants' Assignee's co-pending patents and applications: U.S. Pat. No. 7,125,672, U.S. Pat. No. 7,189,559, U.S. Pat.
20 No. 7,192,762, U.S. Pat. No. 7,198,937, U.S. Pat. No. 7,202,356, U.S. Pat. No. 7,214,491, U.S. Pat. No. 7,238,482, U.S. Pat. No. 7,256,033, U.S. Pat. No. 7,259,255, U.S. Pat. No. 7,264,949, U.S. Pat. No. 7,267,976, U.S. Pat. No. 7,273,746, U.S. Pat. App. No. 10/985254 and No. 10/985691 (filed November 10, 2004), U.S. Pat. App. No. 11/183664 (filed July 18, 2005), U.S.
25 Pat. App. No. 11/185301 (filed July 20, 2005), U.S. Pat. App. No. 11/190750 (filed July 27, 2005), U.S. Pat. App. No. 11/198975 (filed August 8, 2005), U.S. Pat. App. No. 11/253882 (filed October 19, 2005), U.S. Pat. App. No. 11/264784 and No. 11/264737 (filed November 1, 2005), U.S. Pat. App. No. 11/265761 (filed November 2, 2005), U.S. Pat. App. No. 11/601563 and No.
30 11/601564 (filed November 16, 2006), U.S. Pat. App. No. 11/635258 (filed

December 7, 2006), U.S. Pat. App. No. 11/613420 (filed December 20, 2006
U.S. Pat. App. No. 11/787772 (filed April 18, 2007), U.S. Pat. App. No.
11/737772 (filed April 20, 2007), U.S. Pat. App. No. 11/740298 (filed April 26
2007), U.S. Pat. App. No. 12/111237 (filed April 29, 2008), U.S. Pat. App. No
5 11/748629 and No. 11/748637 (filed May 15, 2007), U.S. Pat. App. No.
11/779915 (filed July 19, 2007), U.S. Pat. App. No. 60/991266 (filed
November 30, 2007), U.S. Pat. App. No. 11/952243 (filed December 7, 2007
U.S. Pat. App. No. 61/041716 (filed April 2, 2008), U.S. Pat. App. No.
12/061738 (filed April 3, 2008), U.S. Pat. App. No. 12/099811 (filed April 9,
10 2008), U.S. Pat. App. No. 12/102879 (filed April 15, 2008), U.S. Pat. App. No
12/111237 (filed April 29, 2008), U.S. Pat. App. No. 61/055511 (filed May 23,
2008) and U.S. Pat. App. No. 61/093007 (filed August 29, 2008).

Table 12: Lipid Profile Of Representative *Yarrowia lipolytica* Strains Engineered To Produce ω -3/ ω -6 PUFAs

Strain	Reference	ATCC Deposit No.	Fatty Acid Content (As A Percent [%] of Total Fatty Acids)														Lipid % dcw
			16:0	16:1	18:0	18:1	18:2	18:3 (ALA)	GLA	20:2	DGLA	ARA	ETA	EPA	DPA	DHA	
Wildtype pDMW208 pDMW208D62	US 2006-0035351-A1; WO2006/033723	#76982	14	11	3.5	34.8	31	--	0	--	--	--	--	--	--	--	--
		--	11.9	8.6	1.5	24.4	17.8	--	25.9	--	--	--	--	--	--	--	--
		--	16.2	1.5	0.1	17.8	22.2	--	34	--	--	--	--	--	--	--	--
M4	US 2006-0115881-A1; WO2006/052870	--	15	4	2	5	27	--	35	--	8	0	0	0	--	--	--
		--	13.1	8.1	1.7	7.4	14.8	--	25.2	--	8.3	11.2	--	--	--	--	--
Y2034	US 2006-0094092-A1; WO2006/055322	PTA-7186	15.9	6.6	0.7	8.9	16.6	--	29.7	--	0	10.9	--	--	--	--	--
Y2047		--	7.9	15.3	0	13.7	37.5	--	0	--	7.9	14	--	--	--	--	--
Y2214		--	19	10.3	2.3	15.8	12	--	18.7	--	5.7	0.2	3	10.3	--	--	36
EU	US 2006-0115881-A1; WO2006/052870	--	7.6	4.1	2.2	16.8	13.9	--	27.8	--	3.7	1.7	2.2	15	--	--	--
Y2072		--	9	3	3.5	5.6	18.6	--	29.6	--	3.8	2.8	2.3	18.4	--	--	--
Y2102		--	17	4.5	3	2.5	10	--	20	--	3	2.8	1.7	20	--	--	--
Y2088		--	7.9	3.4	2.5	9.9	14.3	--	37.5	--	2.5	1.8	1.6	17.6	--	--	--
Y2089		--	13	0	2.6	5.1	16	--	29.1	--	3.1	1.9	2.7	19.3	--	--	--
Y2095		--	6	1	6.1	7.7	12.6	--	26.4	--	6.7	2.4	3.6	26.6	--	--	22.9
Y2090		--	8.1	1	6.3	8.5	11.5	--	25	--	5.8	2.1	2.5	28.1	--	--	20.8
Y2096		PTA-7184	11	16.1	0.7	18.4	27	--	--	3.3	3.3	1	3.8	9	--	--	--
Y2201		PTA-7185															
Y3000	US 2006-0110806-A1; WO2006/052871	PTA-7187	5.9	1.2	5.5	7.7	11.7	--	30.1	--	2.6	1.2	1.2	4.7	18.3	5.6	--
Y4001	WO2008/073367	--	4.3	4.4	3.9	35.9	23	0	--	23.8	0	0	0	--	--	--	--
Y4036		--	7.7	3.6	1.1	14.2	32.6	0	--	15.6	18.2	0	0	--	--	--	--
Y4070		--	8	5.3	3.5	14.6	42.1	0	--	6.7	2.4	11.9	--	--	--	--	--
Y4158		--	3.2	1.2	2.7	14.5	30.4	5.3	--	6.2	3.1	0.3	3.4	20.5	--	--	27.3
Y4184		--	3.1	1.5	1.8	8.7	31.5	4.9	--	5.6	2.9	0.6	2.4	28.9	--	--	23.9

Chromosomal Knockout Of Pex Genes

Following selection of a preferred *Yarrowia lipolytica* strain producing the desired ω -3/ ω -6 PUFA (or combination of PUFAs thereof), one of skill in the art could readily engineer a suitable knockout construct, similar to

5 pYPS161 in Example 7, to result in knockout of a chromosomal Pex gene upon transformation into the parental *Y. lipolytica* strain. Preferred Pex gene would include: YIPex1p (GenBank Accession No. CAG82178; SEQ ID NO:1) YIPex2p (GenBank Accession No. CAG77647; SEQ ID NO:2), YIPex3p (GenBank Accession No. CAG78565; SEQ ID NO:3), YIPex3Bp (GenBank

10 Accession No. CAG83356; SEQ ID NO:4), YIPex4p (GenBank Accession No. CAG79130; SEQ ID NO:5), YIPex5p (GenBank Accession No. CAG78803; SEQ ID NO:6), YIPex6p (GenBank Accession No. CAG82306; SEQ ID NO:7), YIPex7p (GenBank Accession No. CAG78389; SEQ ID NO:8), YIPex8p (GenBank Accession No. CAG80447; SEQ ID NO:9), YIPex12p

15 (GenBank Accession No. CAG81532; SEQ ID NO:11), YIPex13p (GenBank Accession No. CAG81789; SEQ ID NO:12), YIPex14p (GenBank Accession No. CAG79323; SEQ ID NO:13), YIPex16p (GenBank Accession No. CAG79622; SEQ ID NO:14), YIPex17p (GenBank Accession No. CAG84025 SEQ ID NO:15), YIPex19p (GenBank Accession No. AAK84827; SEQ ID

20 NO:16), YIPex20p (GenBank Accession No. CAG79226; SEQ ID NO:17), YIPex22p (GenBank Accession No. CAG77876; SEQ ID NO:18) and YIPex26p (GenBank Accession No. NC_006072, antisense translation of nucleotides 117230-118387; SEQ ID NO:19).

It would be expected that the chromosomal disruption of *Pex* would

25 result in an increased amount of PUFAs in the total lipid fraction and in the oi fraction, as a percent of total fatty acids, as compared with a eukaryotic organism whose native peroxisome biogenesis factor protein has not been disrupted, wherein the amount of PUFAs can be: 1) the PUFA that is the desired end product of a functional PUFA biosynthetic pathway, as opposed

30 to PUFA intermediates or by-products, 2) C₂₀ and C₂₂ PUFAs, and/or 3) total PUFAs. Preferred results not only achieve an increase in the amount of

PUFAs as a percent of total fatty acids but also result in an increased amount of PUFAs as a percent of dry cell weight, as compared with a eukaryotic organism whose native peroxisome biogenesis factor protein has not been disrupted. Again, the amount of PUFAs can be: 1) the PUFA that is the
5 desired end product of a functional PUFA biosynthetic pathway, as opposed to PUFA intermediates or by-products, 2) the C₂₀ and C₂₂ PUFAs, and/or 3) the total PUFAs. In some cases, the total lipid content also increases, relative to that of a eukaryotic organism whose native peroxisome biogenesis factor protein has not been disrupted.

10

EXAMPLE 9

Chromosomal Deletion Of Pex16 In *Yarrowia lipolytica* Strain Y4036U Increases Percent DGLA Accumulated

The present Example describes use of construct pYRH13 (FIG. 9B; SEQ ID NO:59) to knock out the chromosomal Pex16 gene in the DGLA-
15 producing *Yarrowia* strain Y4036U (Example 1). Transformation of *Y. lipolytica strain* Y4036U with the Pex16 knockout construct resulted in creation of strain Y4036U (Δ pex16). The effect of the Pex16 knockout on DGLA level was determined and compared. Specifically, knockout of Pex16 resulted in an increased percentage of DGLA as a percent of total fatty acids
20 in the cell.

Construct pYRH13

Plasmid pYRH13 was derived from plasmid pYPS161 (FIG. 9A, SEQ ID NO:56; Example 7). Specifically, a 1982 bp 5' promoter region of the *Yarrowia lipolytica* Pex16 gene (GenBank Accession No. CAG79622)
25 replaced the *Ascl*/*Bs*WI fragment of pYPS161 and a 448 bp 3' terminator region of the *Yarrowia lipolytica* Pex16 gene (GenBank Accession No. CAG79622) replaced the *PacI*/*SphI* fragment of pYPS161 to produce pYRH13 (SEQ ID NO:59; FIG.9B).

Generation Of *Yarrowia lipolytica* Knockout Strain Y4036 (Δ Pex16)

Standard protocols were used to transform *Yarrowia lipolytica* strain Y4036U (Example 1) with the purified 6.0 kB *Ascl/SphI* fragment of Pex16 knockout construct pYRH13.

To screen for cells having the Pex16 deletion, colony PCR was performed using *Taq* polymerase (Invitrogen; Carlsbad, CA) and the PCR primers PEX16Fii (SEQ ID NO:60) and PEX16Rii (SEQ ID NO:61). This set of primers was designed to amplify a 1.1 kB region of the intact Pex16 gene, and therefore the Pex16 deleted mutant (i.e., Δ pex16) would not produce the band. A second set of primers was designed to produce a band only when the Pex16 gene was deleted. Specifically, one primer (i.e., 3UTR-URA3; SEQ ID NO:62) binds to a region in the vector sequences of the introduced 6.0 kB *Ascl/SphI* disruption fragment, and the other primer (i.e., PEX16-conf; SEQ ID NO:63) binds to the Pex16 terminator sequences of chromosome outside of the homologous region of the disruption fragment.

More specifically, the colony PCR was performed using a reaction mixture that contained: 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.5 mM MgCl₂, 400 μ M each of dGTP, dCTP, dATP, and dTTP, 2 μ M of each primer, 20 μ l water and 2 U *Taq* polymerase. Amplification was carried out as follows: initial denaturation at 94 °C for 120 sec, followed by 35 cycles of denaturation at 94 °C for 60 sec, annealing at 55 °C for 60 sec, and elongation at 72 °C for 120 sec. A final elongation cycle at 72 °C for 5 min was carried out, followed by reaction termination at 4 °C.

Of 205 colonies screened, 195 had the Pex16 knockout fragment integrated at a random site in the chromosome and thus were not Δ pex16 mutants (however, the cells could grow on ura- plates, due to the presence of pYRH13). Three of these random integrants, designated as Y4036U-17, Y4036U-19 and Y4036U-33, were used as controls in lipid production experiments (*infra*).

The remaining 10 colonies screened (i.e., of the total 205) contained the Pex16 knockout. These ten Δ pex16 mutants within the Y4036U strain background were designated RHY25 through RHY34.

Confirmation Of *Yarrowia lipolytica* Knockout Strain Y4036U (Δ Pex16) By
 5 Quantitative Real Time PCR

Further confirmation of the Pex16 knockout in strains RHY25 through RHY34 was performed by quantitative real time PCR, with the *Yarrowia* translation elongation factor (tef-1) gene (GenBank Accession No. AF054510) used as the control.

10 First, real time PCR primers and TaqMan probes targeting the Pex16 gene and the tef-1 gene, respectively, were designed with Primer Express software v 2.0 (AppliedBiosystems, Foster City, CA). Specifically, real time PCR primers ef-324F (SEQ ID NO:64), ef-392R (SEQ ID NO:65), PEX16-741F (SEQ ID NO:66) and PEX16-802R (SEQ ID NO:67) were designed, as
 15 well as the TaqMan probes ef-345T (i.e., 5' 6-FAMTM-TGCTGGTGGTGTGAGTT-TAMRATM, wherein the nucleotide sequence is set forth as SEQ ID NO:68) and PEX16-760T (i.e., 5'-6FAMTM-CTGTCCATTCTGCGACCCCTC-TAMRATM, wherein the nucleotide sequence is set forth as SEQ ID NO:69). The 5' end of the TaqMan
 20 fluorogenic probes have the 6FAMTM fluorescent reporter dye bound, while the 3' end comprises the TAMRATM quencher. All primers and probes were obtained from Sigma-Genosys (Woodlands, TX).

Knockout candidate DNA was prepared by suspending 1 colony in 50 μ l of water. Reactions for tef-1 and PEX16 were run separately, in triplicate
 25 for each sample. Real time PCR reactions included 20 pmoles each of forward and reverse primers (i.e., ef-324F, ef-392R, PEX16-741F and PEX16-802R 5', *supra*), 5 pmoles TaqMan probe (i.e., ef-345T and PEX16-760T), 10 μ l TaqMan Universal PCR Master Mix--No AmpErase[®] Uracil-N-Glycosylase (UNG) (Catalog No. PN 4326614, AppliedBiosystems), 1 μ l
 30 colony suspension and 8.5 μ l RNase/DNase free water for a total volume of

20 µl per reaction. Reactions were run on the ABI PRISM® 7900 Sequence Detection System under the following conditions: initial denaturation at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 sec and annealing at 60 °C for 1 min. Real time data was collected automatically during each cycle by monitoring 6-FAM™ fluorescence. Data analysis was performed using tef-1 gene threshold cycle (C_T) values for data normalization as per the ABI PRISM® 7900 Sequence Detection System instruction manual

Based on this analysis, it was concluded that all ten of the Y4036U (Δpex16) colonies (i.e., RHY25 through RHY34) were valid Pex16 knockouts wherein the pYRH13 construct had integrated into the chromosomal YIPex16

Evaluation Of *Yarrowia lipolytica* Strains Y4036U And Y4036U (ΔPex16) For DGLA Production

To evaluate the effect of the Pex16 knockout on the percent of PUFAs in the total lipid fraction and the total lipid content in the cells, the Y4036U and Y4036U (Δpex16) strains were grown under comparable oleaginous conditions. More specifically, strains Y4036U-17, Y4036U-19 and Y4036U-33 having the Pex16 knockout fragment integrated at a random site in the chromosome were considered as Pex16 wild type (i.e., Y4036U) and strains RHY25 through RHY34 were the Pex16 mutant strains (i.e., Y4036U (Δpex16)). Cultures of each strain were grown at a starting OD₆₀₀ of ~0.1 in 25 mL of MM containing 90 mg/L L-leucine in a 125 mL flask for 48 hrs. The cells were harvested by centrifugation for 5 min at 4300 rpm in a 50 mL conical tube. The supernatant was discarded and the cells were re-suspended in 25 mL of HGM and transferred to a new 125 mL flask. The cells were incubated with aeration for an additional 120 hrs at 30 °C.

The fatty acid composition (i.e., LA (18:2), ALA, EDA and DGLA) for each of the strains is shown below in Table 13; fatty acid composition is expressed as the weight percent (wt. %) of total fatty acids. The average fatty acid composition of strains Y4036U and Y4036U (Δpex16) are highlighted in gray and indicated with “Ave”. None of the strains tested

provided sufficient cell mass in MM + L-leucine media, and thus total lipid content was not analyzed.

Table 13

Lipid Composition In *Y. lipolytica* Strains Y4036U And Y4036U (Δ pex16)

Strain	Sample	18:2	ALA	EDA	DGLA
Y4036U	Y4036U-17	26.1	2.4	9.9	24.9
	Y4036U-19	29.4	1.6	9.9	18.1
	Y4036U-33	20.7	3.1	11.2	27.3
Y4036U	AVE	25.4	2.4	10.3	23.4
Y4036U (Δ pex16)	RHY25-1	14.9	5.1	5.5	44.1
	RHY25-2	14.3	5.0	5.4	42.6
	RHY26-1	14.4	5.1	5.6	42.9
	RHY26-2	13.8	4.9	5.9	44.6
	RHY27-1	14.4	5.0	5.4	42.6
	RHY27-2	15.1	4.9	5.6	44.2
	RHY28	15.3	4.6	5.7	42.6
	RHY29	15.4	4.8	5.5	43.9
	RHY30	15.5	4.5	5.9	43.6
	RHY31	15.5	4.7	5.8	43.9
	RHY32	15.5	4.9	5.9	44.4
	RHY33	15.9	4.7	5.9	41.8
	RHY34	15.9	4.9	6.2	43.5
Y4036U (Δ pex16)	AVE	15.1	4.9	5.7	43.4

5

The results in Table 13 showed that knockout of the chromosomal Pex16 gene in Y4036U (Δ pex16) increased the DGLA % TFAs approximately 85%, as compared to the DGLA % TFAs in strain Y4036U whose native Pex16p had not been knocked out. However, Y4036U (Δ pex16) also had a ~40% decrease in the LA (18:2) accumulation.

Thus, the results in Table 13 showed that compared to the parent strain Y4036, Y4036 (Δ Pex16) strain had higher average DGLA % TFAs (43.4% versus 23.4%). Additionally, strain Y4036U (Δ pex16) had a 1.65-fold increase in the amount of DGLA relative to the total PUFAs (62.8% of the PUFAs [as a % TFAs] versus 38.1% of the PUFAs [as a % TFAs]) and a 1.3 fold increase in the amount of C20 PUFAs relative to the total PUFAs (71% of the PUFAs [as a % TFAs] versus 54.8% of the PUFAs [as a % TFAs]).

15

EXAMPLE 10

Generation Of Y4305 Strain To Produce About 53.2% EPA Of Total Lipids

Y. lipolytica strain Y4305U, having a *Ura*- phenotype, was used as the host in Example 11, *infra*. Strain Y4305 (a *Ura*+ strain that was parent to
5 Y4305U) was derived from *Y. lipolytica* ATCC #20362, and is capable of producing about 53.2% EPA relative to the total lipids via expression of a $\Delta 9$ elongase/ $\Delta 8$ desaturase pathway.

The development of strain Y4305U required the construction of strain Y2224, strain Y4001, strain Y4001U, strain Y4036, strain Y4036U, strain
10 Y4070 and strain Y4086 (*supra*, Example 1). Further development of strain Y4305U required construction of strain Y4086U1, strain Y4128 and strain Y4128U3 (*supra*, Example 2). Subsequently, development of strain Y4305U (diagrammed in FIG. 10) required construction of strain Y4217 (producing 42% EPA), strain Y4217U2 (*Ura*-), strain Y4259 (producing 46.5% EPA),
15 strain Y4259U2 (*Ura*-) and strain Y4305 (producing 53.2% EPA).

Although the details concerning transformation and selection of the EPA-producing strains developed after strain Y4128U3 are not elaborated herein, the methodology used for isolation of strain Y4217, strain Y4217U2, strain Y4259, strain Y4259U2, strain Y4305 and strain Y4305U was as
20 described in Examples 1 and 2.

Briefly, construct pZKL2-5U89GC (FIG. 8B; SEQ ID NO:55; described in Table 24 of Int'l. App. Pub. No. WO 2008/073367) was generated to integrate one $\Delta 9$ elongase gene (i.e., EgD9eS), one $\Delta 8$ desaturase gene (i.e. EgD8M), one $\Delta 5$ desaturase gene (i.e., EgD5S), and one *Yarrowia lipolytica*
25 diacylglycerol cholinephosphotransferase (CPT1) gene into the *Lip2* loci (GenBank Accession No. AJ012632) of strain Y4128U3 to thereby enable higher level production of EPA. Six strains, designated as Y4215, Y4216, Y4217, Y4218, Y4219 and Y4220, produced about 41.1%, 41.8%, 41.7%, 41.1%, 41% and 41.1% EPA of total lipids, respectively.

Strain Y4217U1 and Y4217U2 were created by disrupting the *Ura3* gene in strain Y4217 via construct pZKUE3S (FIG. 5A; SEQ ID NO:31; described in Table 22 of Int'l. App. Pub. No. WO 2008/073367), comprising a chimeric EXP1::ME3S::Pex20 gene targeted for the *Ura3* gene. Construct
 5 pZKL1-2SP98C (FIG. 8A; SEQ ID NO:54; described in Table 23 of Int'l. App. Pub. No. WO 2008/073367) was utilized to integrate one $\Delta 9$ elongase gene (i.e., EgD9eS), one $\Delta 8$ desaturase gene (i.e., EgD8M), one $\Delta 12$ desaturase gene (i.e., FmD12S), and one *Yarrowia lipolytica* CPT1 gene into the *Lip1* loc
 10 (GenBank Accession No. Z50020) of strain Y4217U2, thereby resulting in isolation of strains Y4259, Y4260, Y4261, Y4262, Y4263 and Y4264, producing about 46.5%, 44.5%, 44.5%, 44.8%, 44.5% and 44.3% EPA of total lipids, respectively.

A *Ura*- derivative (i.e., strain Y4259U2) was then created, via transformation with construct pZKUM (FIG. 11A; SEQ ID NO:70; described in
 15 Table 33 of Int'l. App. Pub. No. WO 2008/073367) which integrated a *Ura3* mutant gene into the *Ura3* gene of strain Y4259, thereby resulting in isolation of strains Y4259U1, Y4259U2 and Y4259U3, respectively (collectively, Y4259U) (producing 31.4%, 31% and 31.3% EPA of total lipids, respectively).

20 Finally, construct pZKD2-5U89A2 (FIG. 11B; SEQ ID NO:71) was generated to integrate one $\Delta 9$ elongase gene, one $\Delta 5$ desaturase gene, one $\Delta 8$ desaturase gene, and one $\Delta 12$ desaturase gene into the diacylglycerol acyltransferase (*DGAT2*) loci of strain Y4259U2, to thereby enable increased production of EPA. The pZKD2-5U89A2 plasmid contained the following
 25 components:

Table 14

Description of Plasmid pZKD2-5U89A2 (SEQ ID NO:71)

RE Sites And Nucleotides Within SEQ ID NO:71	Description Of Fragment And Chimeric Gene Components

<i>AscI/BsWI</i> (1-736)	728 bp 5' portion of <i>Yarrowia DGAT2</i> gene (SEQ ID NO:72) (labeled as "YLDGAT5" in Figure; U.S. Pat. No. 7,267,976)
<i>PacI/SphI</i> (4164-3444)	714 bp 3' portion of <i>Yarrowia DGAT2</i> gene (SEQ ID NO:72) (labeled as "YLDGAT3" in Figure; U.S. Pat. No. 7,267,976)
<i>SwaI/BsWI</i> (13377-1)	YAT1::FmD12S::Lip2, comprising: • YAT1: <i>Yarrowia lipolytica</i> YAT1 promoter (labeled as "YAT" in Figure; Pat. Appl. Pub. No. US 2006/0094102-A1); • FmD12S: codon-optimized $\Delta 12$ elongase (SEQ ID NO:74), derived from <i>Fusarium moniliforme</i> (labeled as "F.D12S" in Figure; Int'l. App. Pub. No. WO 2005/047485); • Lip2: Lip2 terminator sequence from <i>Yarrowia Lip2</i> gene (GenBank Accession No. AJ012632)
<i>PmeI/SwaI</i> (10740-13377)	FBAIN::EgD8M::Lip1 comprising: • FBAIN: <i>Yarrowia lipolytica</i> FBAIN promoter (U.S. Pat. No. 7,202,356); • EgD8M: Synthetic mutant $\Delta 8$ desaturase (SEQ ID NO:76; Pat. Appl. Pub. No. US 2008-0138868 A1), derived from <i>Euglena gracilis</i> ("EgD8S"; U.S. Pat. No. 7,256,033); • Lip1: Lip1 terminator sequence from <i>Yarrowia Lip1</i> gene (GenBank Accession No. Z50020)
<i>Clal/PmeI</i> (8846-10740)	YAT1::E389D9eS::OCT, comprising: • YAT1: <i>Yarrowia lipolytica</i> YAT1 promoter (labeled as "YAT" in Figure; Pat. Appl. Pub. No. US 2006/0094102-A1); • E389D9eS: codon-optimized $\Delta 9$ elongase (SEQ ID NO:78), derived from <i>Eutreptiella</i> sp. CCMP389 (labeled as "D9ES-389" in Figure; Int'l. App. Pub. No. WO 2007/061742); • OCT: OCT terminator sequence from <i>Yarrowia OCT</i> gene (GenBank Accession No. X69988)
<i>Clal/EcoRI</i> (8846-6777)	<i>Yarrowia Ura3</i> gene (GenBank Accession No. AJ306421)
<i>EcoRI/PacI</i> (6777-4164)	EXP1::EgD5S::ACO, comprising: • EXP1: <i>Yarrowia lipolytica</i> export protein (EXP1) promoter (labeled as "Exp" in Figure; Int'l. App. Pub. No. WO 2006/052870); • EgD5S: codon-optimized $\Delta 5$ desaturase (SEQ ID NO:80), derived from <i>Euglena gracilis</i> (Pat. Appl. Pub. No. US 2007-0292924-A1); • Aco: Aco terminator sequence from <i>Yarrowia Aco</i>

	gene (GenBank Accession No. AJ001300)
--	---------------------------------------

The pZKD2-5U89A2 plasmid was digested with *Ascl*/*Sph*I and then used for transformation of strain Y4259U2 according to the General Methods. The transformed cells were plated onto MM plates, and plates were
5 maintained at 30 °C for 3 to 4 days. Single colonies were re-streaked onto MM plates, and the resulting colonies were used to inoculate liquid MM. Liquid cultures were shaken at 250 rpm/min for 2 days at 30 °C. The cells were collected by centrifugation, resuspended in HGM, and then shaken at 250 rpm/min for 5 days. The cells were collected by centrifugation, and lipids
10 were extracted. FAMES were prepared by trans-esterification and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed that most of the selected 96 strains produced 40-46% EPA of total lipids. Four strains, designated as Y4305, Y4306, Y4307 and Y4308, produced about 53.2%, 46.4%, 46.8% and 47.8% EPA of total
15 lipids, respectively. The complete lipid profile of Y4305 is as follows: 16:0 (2.8%), 16:1 (0.7%), 18:0 (1.3%), 18:1 (4.9%), 18:2 (17.6%), ALA (2.3%), EDA (3.4%), DGLA (2.0%), ARA (0.6%), ETA (1.7%) and EPA (53.2%). The total lipid % dry cell weight was 27.5.

The final genotype of strain Y4305 with respect to wild type *Yarrowia*
20 *lipolytica* ATCC #20362 was SCP2- (YALI0E01298g), YALI0C18711g-, Pex10-, YALI0F24167g-, *unknown 1*-, *unknown 3*-, *unknown 8*-, GPD::FmD12::Pex20, YAT1::FmD12::OCT, GPM/FBAIN::FmD12S::OCT, EXP1::FmD12S::Aco, YAT1::FmD12S::Lip2, YAT1::ME3S::Pex16, EXP1::ME3S::Pex20 (3 copies), GPAT::EgD9e::Lip2, EXP1::EgD9eS::Lip1,
25 FBAINm::EgD9eS::Lip2, FBA::EgD9eS::Pex20, GPD::EgD9eS::Lip2, YAT1::EgD9eS::Lip2, YAT1::E389D9eS::OCT, FBAINm::EgD8M::Pex20, FBAIN::EgD8M::Lip1 (2 copies), EXP1::EgD8M::Pex16, GPDIN::EgD8M::Lip1, YAT1::EgD8M::Aco, FBAIN::EgD5::Aco, EXP1::EgD5S::Pex20, YAT1::EgD5S::Aco, EXP1::EgD5S::ACO,

YAT1::RD5S::OCT, YAT1::PaD17S::Lip1, EXP1::PaD17::Pex16,
FBAINm::PaD17::Aco, YAT1::YICPT1::ACO, GPD::YICPT1::ACO.

In order to disrupt the *Ura3* gene in strain Y4305, construct pZKUM (FIG. 11A; SEQ ID NO:70; described in Table 33 of Int'l. App. Pub. No. WO 2008/073367) was used to integrate a *Ura3* mutant gene into the *Ura3* gene of strain Y4305. A total of 8 transformants grown on MM + 5-FOA plates were picked and re-streaked onto MM plates and MM + 5-FOA plates, separately. All 8 strains had a *Ura*- phenotype (i.e., cells could grow on MM 5-FOA plates, but not on MM plates). The cells were scraped from the MM + 5-FOA plates, and lipids were extracted. FAMES were prepared by trans-esterification and subsequently analyzed with a Hewlett-Packard 6890 GC.

GC analyses showed the presence of 37.6%, 37.3% and 36.5% EPA of total lipids in pZKUM transformants #1, #6 and #7 grown on MM + 5-FOA plates. These three strains were designated as strains Y4305U1, Y4305U2 and Y4305U3, respectively (collectively, Y4305U). For clarity in Example 11, strain Y4305U is referred to as strain Y4305U (Δ pex10).

EXAMPLE 11

Chromosomal Deletion Of Pex16 In *Yarrowia lipolytica* Strain Y4305U (Δ pex10) Further Increased Percent EPA Accumulated (The Double Pex10-Pex16 Knockout)

The present Example describes use of construct pYRH13 (FIG. 9B; SEQ ID NO:59) to knock out the chromosomal Pex16 in *Yarrowia* strain Y4305U (Δ pex10) (Example 10), to thereby result in a Pex10-Pex16 double mutant. The effect of the Pex10-Pex16 double knockout on total oil and EPA level was determined and compared. Specifically, the effect of the Pex10-Pex16 double mutation in strain Y4305U (Δ pex10) (Δ pex16) resulted in an increased amount of EPA in the cell (EPA % TFAs and EPA % DCW), as compared to the single mutant (i.e., strain Y4305U (Δ pex10)).

Generation Of *Yarrowia lipolytica* Knockout Strain Y4305U (Δ pex10) (Δ pex16)

Standard protocols were used to transform *Yarrowia lipolytica* strain Y4305U (Δ pex10) (Example 10) with the purified 6.0 kB *Ascl*/*SphI* fragment of Pex16 knockout construct pYRH13 (Example 9; SEQ ID NO:59).

Screening and identification of cells having the Pex16 deletion was conducted by colony PCR, as described in Example 9.

Of 93 colonies screened, 88 had the Pex16 knockout fragment integrated at a random site in the chromosome and thus were not Δ pex16 mutants (however, the cells could grow on Ura- plates, due to the presence of pYRH13). Two of these random integrants, designated as Y4305U-22 and Y4305U-25, were used as controls in lipid production experiments (*infra*).

The remaining 5 colonies screened (i.e., of the total 93) contained the Pex16 knockout. These five Δ pex16 mutants within the Y4305U strain background were designated RHY20, RHY21, RHY22, RHY23 and RHY24. Further confirmation of the YIPex16 knockout was performed by quantitative real time PCR, as described in Example 9.

Evaluation Of *Yarrowia lipolytica* Strains Y4305U (Δ Pex10) And Y4305U (Δ Pex10) (Δ pex16) For EPA Production

To evaluate the effect of mutation in multiple Pex genes on the percent of PUFAs in the total lipid fraction and the total lipid content in the cells, Y4305U (Δ pex10) and Y4305U (Δ pex10) (Δ pex16) strains were grown under comparable oleaginous conditions. More specifically, strains Y4305U-22 and Y4305U-25 having the Pex16 knockout fragment integrated at a random site in the chromosome were considered as Pex16 wild type, Pex10 knockouts (i.e., Y4305U (Δ pex10)). Strains RHY22, RHY23 and RHY24 were the double knockout mutant strains (i.e., Y4305U (Δ pex10) (Δ pex16)). Cultures of each strain were grown in duplicate under comparable oleaginous conditions.

Specifically, cultures were grown at a starting OD₆₀₀ of ~0.1 in 25 mL of synthetic dextrose media (SD) in a 125 mL flask for 48 hrs. The cells were harvested by centrifugation for 5 min at 4300 rpm in a 50 mL conical tube. The supernatant was discarded and the cells were re-suspended in 25 mL of

HGM and transferred to a new 125 mL flask. The cells were incubated with aeration for an additional 120 hrs at 30 °C.

To determine the dry cell weight (DCW), the cells from 5 mL of the HGM-grown cultures were processed. The cultured cells were centrifuged for 5 min at 4300 rpm. The pellet was re-suspended using 10 mL of sterile water and was centrifuged under the same conditions for a second time. The pellet was then re-suspended using 1 mL of sterile H₂O (three times) and was transferred to a pre-weighed aluminum pan. The cell suspension was dried overnight in a vacuum oven at 80 °C. The weight of the cells was determined.

To determine the total lipid content, 1 mL of HGM cultured cells were collected by centrifugation for 1 min at 13,000 rpm, total lipids were extracted and FAMES were prepared by trans-esterification, and subsequently analyzed with a Hewlett-Packard 6890 GC (General Methods).

The fatty acid composition (i.e., 16:0 (palmitate), 16:1 (palmitoleic acid), 18:0, 18:1 (oleic acid), 18:2 (LA), 18:3 (ALA), EDA, DGLA, ARA, ETrA, ETA and EPA) for each of the strains is shown below in Table 15 (expressed as the weight percent (wt. %) of total fatty acids (TFA)), as well as the DCW (g/L and total lipid content (TFAs %DCW). The average fatty acid composition of strains Y4305U (Δ pex10) and Y4305U (Δ pex10) (Δ pex16) are highlighted in gray and indicated with "Ave".

Table 15
Lipid Composition In *Y. lipolytica* Strains Y4305U (Δ Pex10) And Y4305U (Δ Pex10) (Δ Pex16)

Strain	Sample	DCW (g/L)	TFAs % DCW	16:0	16:1	18:0	18:1	18:2	18:3	EDA	DGLA	ARA	ETrA	ETA	EPA
Y4305U (Δ pex10)	Y4305U -22 #1	3.50	29	3.1	0.7	2.1	6.4	18.7	2.6	4.2	1.8	0.5	1.8	2.0	45.4
	Y4305U -22 #2	3.94	29	3.0	0.7	2.1	6.2	18.5	2.5	4.5	1.8	0.5	1.8	2.0	45.6
	Y4305U -25 #1	4.14	31	3.6	1.1	1.8	6.1	18.8	2.4	4.5	1.9	0.6	1.6	2.0	43.9
	Y4305U -25 #2	4.12	30	3.6	1.1	1.8	6.1	18.7	2.4	4.6	1.9	0.6	1.6	2.0	44.0
Y4305U (Δ pex10)	AVE	3.93	30	3.3	0.9	2.0	6.2	18.7	2.5	4.5	1.9	0.6	1.7	2.0	44.7
Y4305U (Δ pex10) (Δ pex16)	RHY22 #1	4.04	29	2.7	0.7	1.5	5.4	18.5	2.7	3.4	1.9	0.5	1.4	2.0	48.5
	RHY22 #2	3.82	32	2.7	0.6	1.5	5.5	18.4	3.0	3.0	2.0	0.5	1.5	2.0	48.8
	RHY23 #1	4.66	30	2.7	0.7	1.5	5.4	18.6	2.7	3.5	2.0	0.6	1.4	2.0	48.2
	RHY23 #2	4.18	30	2.7	0.7	1.5	5.4	18.4	2.6	3.5	1.9	0.6	1.4	2.0	48.5
	RHY24 #1	4.34	30	2.8	0.8	1.5	5.5	18.6	2.6	3.6	1.9	0.6	1.4	2.0	47.9
	RHY24 #2	4.58	30	2.7	0.7	1.5	5.6	18.8	2.6	3.6	2.0	0.6	1.4	2.0	47.8
Y4305U (Δ pex10) (Δ pex16)	AVE	4.27	30	2.7	0.7	1.5	5.5	18.6	2.7	3.4	2.0	0.6	1.4	2.0	48.3

The results in Table 15 showed that knockout of the chromosomal Pex16 gene in Y4305U (Δ pex10) (Δ pex16) increased the EPA % TFAs approximately 8%, as compared to the EPA % TFAs in strain Y4305U (Δ pex10) whose native Pex16p had not been knocked out. Additionally, the EPA % DCW was also increased in the double mutant as compared to in the single mutant strain, while the TFAs % DCW remained the same.

Thus, the results in Table 15 showed that compared to the control Y4305 (Δ Pex10) strains, Y4305 (Δ Pex10, Δ Pex16) strains on average had higher EPA % TFAs (48.3% versus 44.7%) and higher EPA % DCW (14.57% versus 13.23%). Strain Y4305 (Δ Pex10, Δ Pex16) had only a 1.05-fold increase in the amount of EPA relative to the total PUFAs (61% of the PUFAs [as a % TFAs] versus 58.3% of the PUFAs [as a % TFAs]) relative to strain Y4305 (Δ Pex10), while the increase in the amount of C20 PUFAs relative to the total PUFAs was effectively identical (73% of the PUFAs [as a % TFAs] versus 72% of the PUFAs [as a % TFAs]).

EXAMPLE 12

Chromosomal Deletion Of Pex3 In *Yarrowia lipolytica* Strain Y4036U Increases Percent DGLA Accumulated

The present Example describes use of construct pY157 (FIG. 12B; SEQ ID NO:82) to knock out the chromosomal Pex3 gene (SEQ ID NO:3) in the Ura⁻, DGLA-producing *Yarrowia* strain Y4036U (Example 1).

Transformation of *Y. lipolytica* strain Y4036U with the Pex3 knockout construct resulted in creation of strain Y4036 (Δ pex3). The effect of the Pex3 knockout on DGLA level was determined and compared to the control strain Y4036 (a Ura⁺ strain that was parent to strain Y4036U). Specifically, knockout of Pex3 increased DGLA as a percentage of total fatty acids and improved ca. 3-fold DGLA % DCW, compared to the control.

Construct pY157

Plasmid pY87 (FIG. 12A) contained a cassette to knock out the *Yarrowia lipolytica* diacylglycerol acyltransferase (*DGAT2*) gene, as describe below in Table 16:

Table 16

5	<u>Description of Plasmid pY87 (SEQ ID NO:83)</u>	
	RE Sites And Nucleotides Within SEQ ID NO:83	Description Of Fragment And Chimeric Gene Components
	<i>SphI/PacI</i> (1-721)	5' portion of <i>Yarrowia DGAT2</i> gene (bases 1-720 of SEQ NO:72) (U.S. Patent 7,267,976)
	<i>PacI/BglII</i> (721-2459)	LoxP:: <i>Ura3</i> ::LoxP, comprising: • LoxP sequence (SEQ ID NO:84); • <i>Yarrowia Ura3</i> gene (GenBank Accession No. AJ306421) • LoxP sequence (SEQ ID NO:84)
	<i>BglII/AscI</i> (2459-3203)	3' portion of <i>Yarrowia DGAT2</i> gene (bases 2468-3202 of SEQ ID NO:72) (U.S. Patent 7,267,976)
	<i>AscI/SphI</i> (3203-5910)	Vector backbone including: • <i>ColE1</i> plasmid origin of replication; • ampicillin-resistance gene (Amp ^R) for selection in <i>E. coli</i> (4191-5051); • <i>E. coli</i> f1 origin of replication

Plasmid pY157 was derived from plasmid pY87. Specifically, a 704 bp 5' promoter region of the *Yarrowia lipolytica* Pex3 gene replaced the *SphI/PacI* fragment of pY87 and a 448 bp 3' terminator region of the *Yarrowia lipolytica* Pex3 gene replaced the *BglII/AscI* fragment of pY87 to produce pY157 (SEQ ID NO:82; FIG. 12B).

Generation Of *Yarrowia lipolytica* Knockout Strain Y4036 (Δ Pex3)

Standard protocols were used to transform *Yarrowia lipolytica* strain Y4036U (Example 1) with the purified 3648 bp *AscI/SphI* fragment of Pex3 knockout construct pY157 (*supra*).

To screen for cells having the Pex3 deletion, colony PCR was performed using *Taq* polymerase (Invitrogen; Carlsbad, CA) and the PCR primers UP 768 (SEQ ID NO:85) and LP 769 (SEQ ID NO:86). This set of primers was designed to amplify a 2039 bp wild type band of the intact Pex3

gene and 3719 bp knockout-specific band when the Pex3 gene was disrupted by targeted knockout.

More specifically, the colony PCR was performed using a MasterAmp Taq kit (Epicentre Technologies, Madison, WI; Catalog No. 82250) and the manufacturer's instructions in a 25 μ l reaction comprising: 2.5 μ l of 10X MasterAmp Taq buffer, 2.0 μ l of 25 mM MgCl₂, 7.5 μ l of 10X MasterAmp Enhancer, 2.5 μ l of 2.5 mM dNTPs (TaKaRa Bio Inc., Otsu Shiga, Japan), 1.0 μ l of 10 μ M Upper primer, 1.0 μ l of 10 μ M Lower primer, 0.25 μ l of MasterAmp Taq DNA polymerase and 19.75 μ l of water. Amplification was carried out as follows: initial denaturation at 95 °C for 5 min, followed by 40 cycles of denaturation at 95 °C for 30 sec, annealing at 56 °C for 60 sec, and elongation at 72 °C for 4 min. A final elongation cycle at 72 °C for 10 min was carried out, followed by reaction termination at 4 °C.

Of 48 colonies screened, 46 had the 2039 bp band expected from the wild type (i.e., undisrupted) Pex3 gene thus were not Δ pex3 mutants. The remaining 2 colonies showed only a faint band of 2039 bp, suggesting that they were Δ pex3 mutants with some contaminating untransformed cells present in the background. This was confirmed by streaking the 2 putative knockout colonies on selection plates to isolate single colonies. Then, genomic DNA was isolated from 3 single colonies from each putative knockout strain and screened by the same primer pair. i.e., UP 768 and LP 769 (SEQ ID NOs:85 and 86). This method was considered more sensitive than colony PCR. All three single colonies from both primary transformants lacked the 2039 bp wild type band and instead possessed the 3719 bp knockout-specific band. The two Δ pex3 mutants within the Y4036U strain background were designated L134 and L135.

Evaluation Of *Yarrowia lipolytica* Strains Y4036 And Y4036 (Δ Pex3) For DGLA Production

To evaluate the effect of the Pex3 knockout on the percent of PUFAs in the total lipid fraction and the total lipid content in the cells, the Y4036 and

Y4036 (Δ pex3) strains were grown under comparable oleaginous conditions. Strains Y4036, L134 (i.e., Y4036 (Δ pex3)) and L135 (i.e., Y4036 (Δ pex3)) were inoculated into 25 mL of CSM-Ura and grown at 30 °C overnight in a shaker. The preculture was aliquoted into fresh 25 mL CSM-Ura flasks at a

5 final OD₆₀₀ of 0.4. Cultures were grown at 30 °C in shaker. After 48 hrs, the cells (which barely grew) were spun down and resuspended in fresh 25 mL CSM-Ura and continued to grow for 72 hrs. Cells were spun down, re-suspended in 25 mL of HGM, and continued to grow as above for 72 hrs. Cells were harvested by centrifugation, washed once in distilled water and

10 resuspended in 25 mL water to give a final volume of 20.5 mL. An aliquot (1.5 mL) was used for lipid content, following extraction of the total lipids, preparation of FAMES by base trans-esterification, and analysis by a Hewlett-Packard 6890 GC (General Methods). The remaining aliquot was dried down to measure dry cell weight (DCW), as described in Example 11.

15 The fatty acid composition (i.e., 16:0 (palmitate), 16:1 (palmitoleic acid), 18:0, 18:1 (oleic acid), 18:2 (LA), EDA and DGLA) for each of the strains is shown below in Table 17 (expressed as the weight percent (wt. %) of total fatty acids (TFA)), as well as the total lipid content (TFA %DCW). The conversion efficiency ("CE") was measured according to the following

20 formula: $([\text{product}]/[\text{substrate}+\text{product}]) \times 100$, where 'product' includes the immediate product and all products in the pathway derived from it. Thus, the Δ 12 desaturase conversion efficiency (Δ 12 % CE) was calculated as: $([\text{LA}+\text{EDA}+\text{DGLA}]/[18:1+\text{LA}+\text{EDA}+\text{DGLA}]) \times 100$; the Δ 9 elongase conversion efficiency (Δ 9 elo % CE) was calculated as:

25 $([\text{EDA}+\text{DGLA}]/[\text{LA}+\text{EDA}+\text{DGLA}]) \times 100$; and, the Δ 8 desaturase conversion efficiency (Δ 8 % CE) was calculated as: $([\text{DGLA}]/[\text{EDA}+\text{DGLA}]) \times 100$. The average fatty acid composition of strains Y4036, L134 and L135 are highlighted in gray and indicated with "Ave", while "S.D." indicates the Standard Deviation. As expected, the Δ pex3 strains did not grow on plates

30 with oleate as a sole source of carbon.

Table 17
Lipid Content And Composition In *Y. lipolytica* Strains Y4036 And Y4036 (Δ Pex3)

Strain	Sample	TFA % DCW	16:0	16:1	18:0	18:1	18:2	EDA	DGLA	Δ 12 % CE	Δ 9 elo % CE	Δ 8 % CE
Y4036	Y4036-1	6.1	10	7	1	14	29	9	19	80	49	69
	Y4036-2	3.7	11	6	1	14	30	8	20	81	48	70
	Y4036-3	4.1	11	5	1	15	31	8	19	80	47	70
	Avg.	4.7	10	6	1	14	30	8	19	80	48	70
	S.D.	1.3	0.3	0.9	0.1	0.3	0.7	0.3	0.2	0.3	0.9	0.8
Y4036 (Δ pex3)	L134-1	6.2	7	5	1	8	12	10	45	89	83	81
	L134-2	5.4	7	5	1	8	11	10	47	90	83	82
	L134-3	6.7	6	5	1	8	12	11	46	90	83	82
	Avg.	6.1	7	5	1	8	12	10	46	90	83	82
	S.D.	0.6	0.5	0.1	0.1	0.3	0.1	0.1	1.0	0.5	0.4	0.3
Y4036 (Δ pex3)	L135-1	4.2	7	5	1	8	12	11	45	89	82	81
	L135-2	6.5	6	5	1	8	12	10	47	90	83	82
	L135-3	7.1	7	5	1	8	12	10	46	90	83	82
	Avg.	5.9	7	5	1	8	12	10	46	90	83	81
	S.D.	1.6	0.6	0.1	0.1	0.3	0.1	0.1	1.1	0.5	0.4	0.5

The results in Table 17 showed that knockout of the chromosomal Pex3 gene in Y4036 (Δ pex3) increased the DGLA % TFAs approximately 142%, as compared to the DGLA % TFAs in strain Y4036 whose native Pex3p had not been knocked out. Specifically, the Pex3 knockout increased DGLA levels from ca. 19% in Y4036 to 46% in Y4036 (Δ pex3) strains, L134 and L135. Additionally, the Δ 9 elongase percent conversion efficiency increased from ca. 48% in Y4036 to 83% in Y4036 (Δ pex3) strains, L134 and L135; and, TFA % DCW increased from 4.7% to 6% in the strains L134 and L135. The LA % TFAs decreased from 30% to 12%. Pex3 deletion indeed increases the flux of fatty acids and thus the substrate availability for Δ 9 elongation.

Thus, the results in Table 17 showed that compared to the parent strain Y4036, Y4036 (Δ Pex3) strain had on average higher lipid content (TFAs % DCW) (ca. 6.0% versus 4.7%), higher DGLA % TFAs (46% versus 19%), and higher DGLA % DCW (ca. 2.8% versus 0.9%). Additionally, strain Y4036 (Δ Pex3) had a 2-fold increase in the amount of DGLA relative to the total PUFAs (67.7% of the PUFAs [as a % TFAs] versus 33.3% of the PUFAs [as a % TFAs]) and a 1.7-fold increase in the amount of C20 PUFAs relative to the total PUFAs (82% of the PUFAs [as a % TFAs] versus 47% of the PUFAs [as a % TFAs]).

It is hypothesized that the improved DGLA productivity would also result in improved EPA productivity in *Yarrowia lipolytica* strains engineered for EPA production (e.g., *Y. lipolytica* strain Y4305U, as described in Example 10, and derivatives therefrom).

25

CLAIMS

What is claimed is:

1. A method of increasing the weight percent of at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in an oleaginous eukaryotic organism having a total lipid content, a total lipid fraction and an oil fraction, comprising:
 - a) providing an oleaginous eukaryotic organism comprising:
 - 1) genes encoding a functional polyunsaturated fatty acid biosynthetic pathway; and
 - 2) a disruption in a native gene encoding a peroxisome biogenesis factor protein, thereby providing a PEX-disrupted organism, and
 - b) growing the PEX-disrupted organism under conditions as to increase the weight percent of at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in the total lipid fraction or in the oil fraction, when compared to the weight percent of the at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids in the total lipid fraction or in the oil fraction in the oleaginous eukaryotic organism in which no native gene encoding a peroxisome biogenesis factor protein has been disrupted.
2. A method of increasing the percent of at least one polyunsaturated fatty acid relative to the dry cell weight in an oleaginous eukaryotic organism, comprising:
 - a) providing an oleaginous eukaryotic organism comprising:
 - 1) genes encoding a functional polyunsaturated fatty acid biosynthetic pathway; and
 - 2) a disruption in a native gene encoding a peroxisome

biogenesis factor protein, thereby providing a PEX-disrupted organism, and

b) growing the PEX-disrupted organism under conditions as to increase the percent of at least one polyunsaturated fatty acid relative to the dry cell weight, when compared to the percent of the at least one polyunsaturated fatty acid relative to the dry cell weight in the oleaginous eukaryotic organism in which no native gene encoding a peroxisome biogenesis factor protein has been disrupted.

3. The method of Claim 1 or 2, wherein the increase in the weight percent of the at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids is at least 1.3, when compared to the weight percent of polyunsaturated fatty acids relative to the weight percent of total fatty acids in the total lipid fraction or in the oil fraction in an oleaginous eukaryotic organism in which no native gene encoding a peroxisome biogenesis factor protein has been disrupted.

4. The method of Claim 1 or 2, wherein the at least one polyunsaturated fatty acid is selected from the group consisting of: linoleic acid, conjugated linoleic acid, γ -linolenic acid, dihomo- γ -linolenic acid, arachidonic acid, docosatetraenoic acid, ω -6 docosapentaenoic acid, α -linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, ω -3 docosapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, docosahexaenoic acid, hydroxylated or epoxy fatty acids of these, C₁₈ polyunsaturated fatty acids, C₂₀ polyunsaturated fatty acids, and C₂₂ polyunsaturated fatty acids.

5. The method of Claim 1, wherein the at least one polyunsaturated fatty acid consists of a combination of polyunsaturated fatty acids and wherein the

weight percent of the combination is increased relative to the weight percent of total fatty acids.

6. The method of Claim 4, wherein the combination of polyunsaturated fatty acids consists of any combination of two or more polyunsaturated fatty acids selected from the group consisting of:

linoleic acid, conjugated linoleic acid, γ -linolenic acid, dihomo- γ -linolenic acid, arachidonic acid, docosatetraenoic acid, ω -6 docosapentaenoic acid, α -linolenic acid, stearidonic acid, eicosatetraenoic acid, eicosapentaenoic acid, ω -3 docosapentaenoic acid, eicosadienoic acid, eicosatrienoic acid, docosahexaenoic acid, hydroxylated or epoxy fatty acids of these, a combination of C₂₀ polyunsaturated fatty acids, a combination of C₂₀₋₂₂ polyunsaturated fatty acids, and a combination of C₂₂ polyunsaturated fatty acids.

7. The method of Claim 1 or 2, wherein the total lipid content in the PEX-disrupted organism is altered, when compared with the total lipid content in an oleaginous eukaryotic organism in which no native gene encoding a peroxisome biogenesis factor protein has been disrupted.

8. The method of Claim 1 or 2, wherein the PEX-disrupted organism is selected from the group consisting of: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon*, *Lipomyces*, *Mortierella*, *Thraustochytrium*, *Schizochytrium*, and *Saccharomyces* having the property of oleaginity.

9. The method of Claim 1 or 2, wherein the polyunsaturated fatty acid biosynthetic pathway comprises genes encoding enzymes selected from the group consisting of:

$\Delta 9$ desaturase, $\Delta 12$ desaturase, $\Delta 6$ desaturase, $\Delta 5$ desaturase, $\Delta 17$ desaturase, $\Delta 8$ desaturase, $\Delta 15$ desaturase, $\Delta 4$ desaturase, $C_{14/16}$ elongase, $C_{16/18}$ elongase, $C_{18/20}$ elongase, $C_{20/22}$ elongase and $\Delta 9$ elongase.

10. The method of Claim 1 or 2, wherein the disruption in the native gene encoding a peroxisome biogenesis factor protein comprises a deletion selected from the group consisting of:

a deletion in a portion of the gene encoding the C-terminal portion of the protein and a gene knockout.

11. The method of Claim 1 or 2, wherein the peroxisome biogenesis factor protein is selected from the group consisting of:

Pex1p, Pex 2p, Pex3p, Pex3Bp, Pex4p, Pex5p, Pex5Bp, Pex5Cp, Pex5/20p, Pex6p, Pex7p, Pex8p, Pex10p, Pex12p, Pex13p, Pex14p, Pex15p, Pex16p, Pex17p, Pex14/17p, Pex18p, Pex19p, Pex20p, Pex21p, Pex21Bp, Pex22p, Pex22p-like and Pex26p.

12. The method of Claim 11, wherein the peroxisome biogenesis factor wherein the disruption is a gene knockout or . a deletion in a portion of the gene encoding the C-terminal portion of the C_3HC_4 zinc ring finger motif of the protein.

13. The oil fraction or the total lipid fraction in a PEX-disrupted organism having an increase in the weight percent of at least one polyunsaturated fatty acid relative to the weight percent of total fatty acids, wherein the increase was obtained by the method of Claim 1.

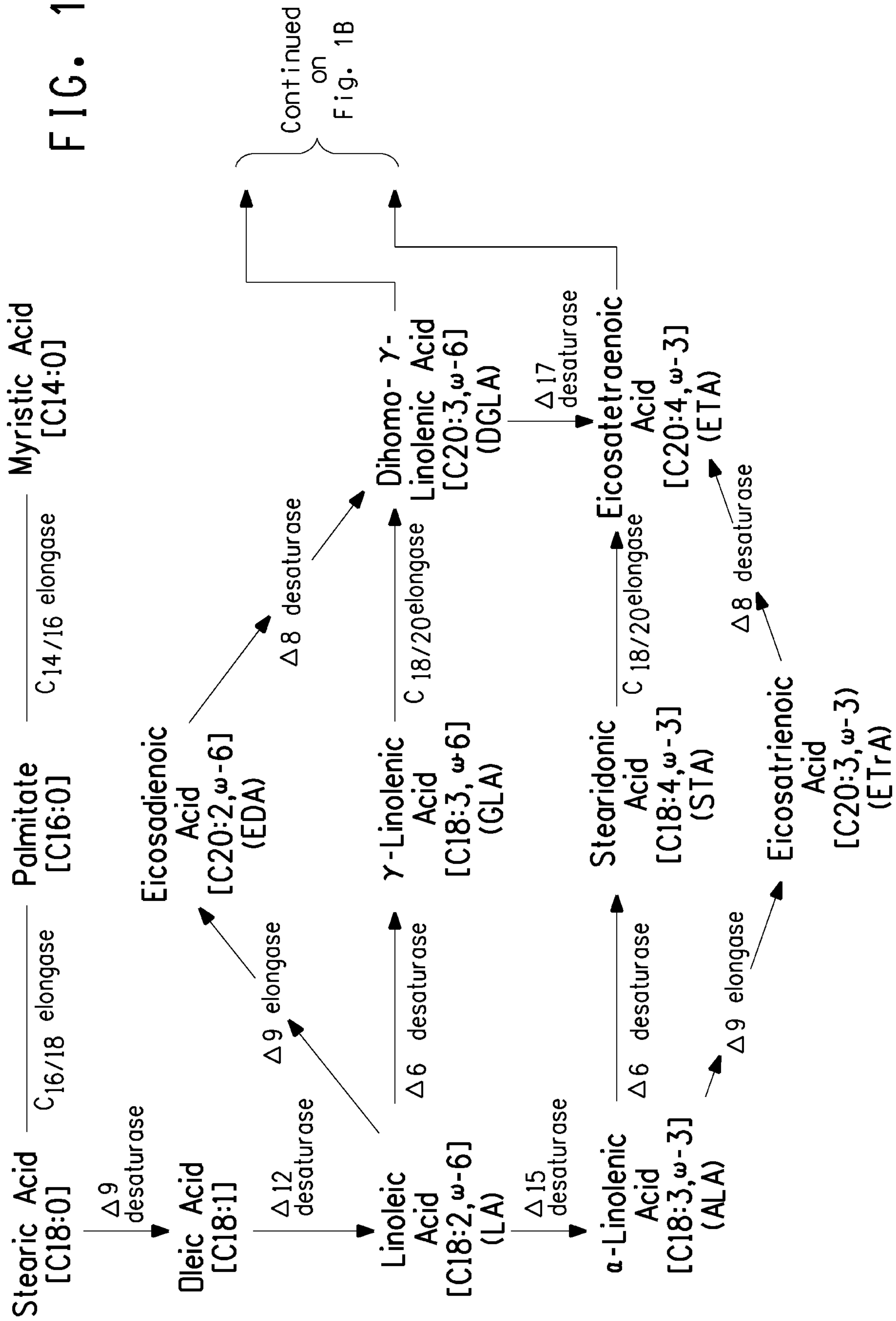
14. Use as food, feed or in an industrial application of the at least one polyunsaturated fatty acid of a PEX-disrupted organism having been

increased in weight percent relative to the weight percent of total fatty acids by the method of Claim 1.

15. A PEX-disrupted *Yarrowia lipolytica*, wherein the disruption occurs in the native gene encoding a peroxisome biogenesis factor protein selected from the group consisting of Pex3p, Pex10p and Pex16p.

1/16

FIG. 1A



2/16

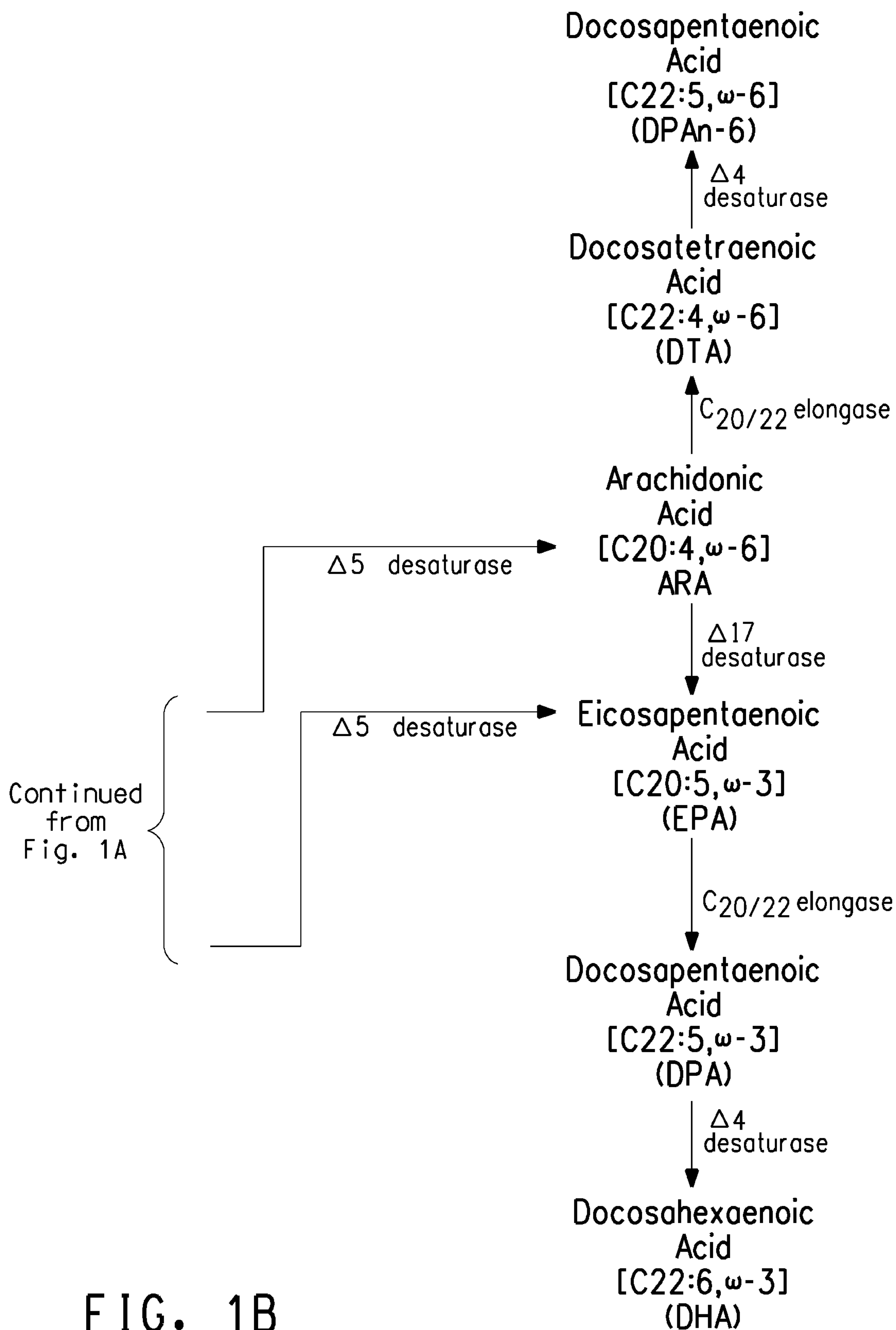


FIG. 2A

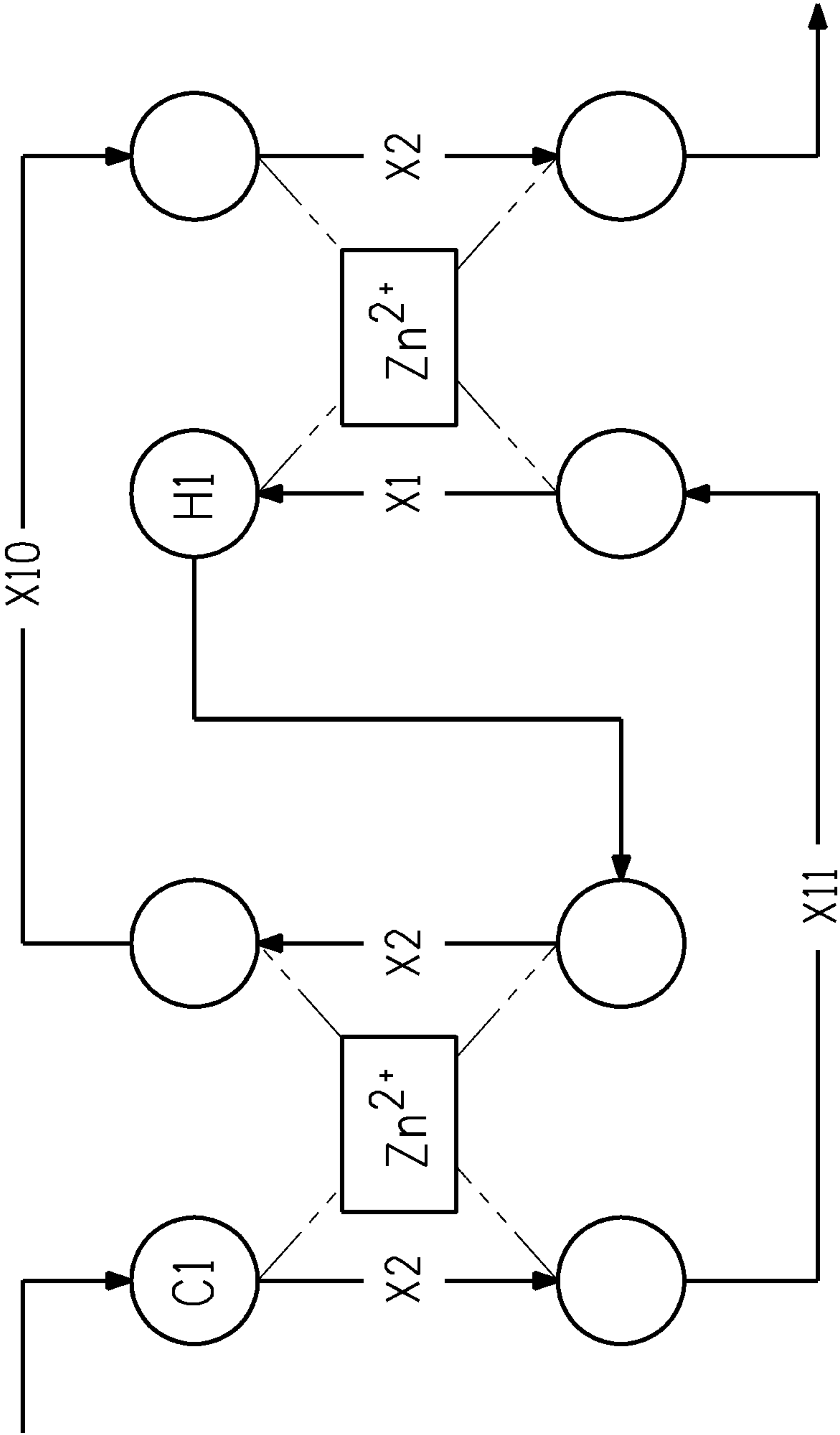


FIG. 2B

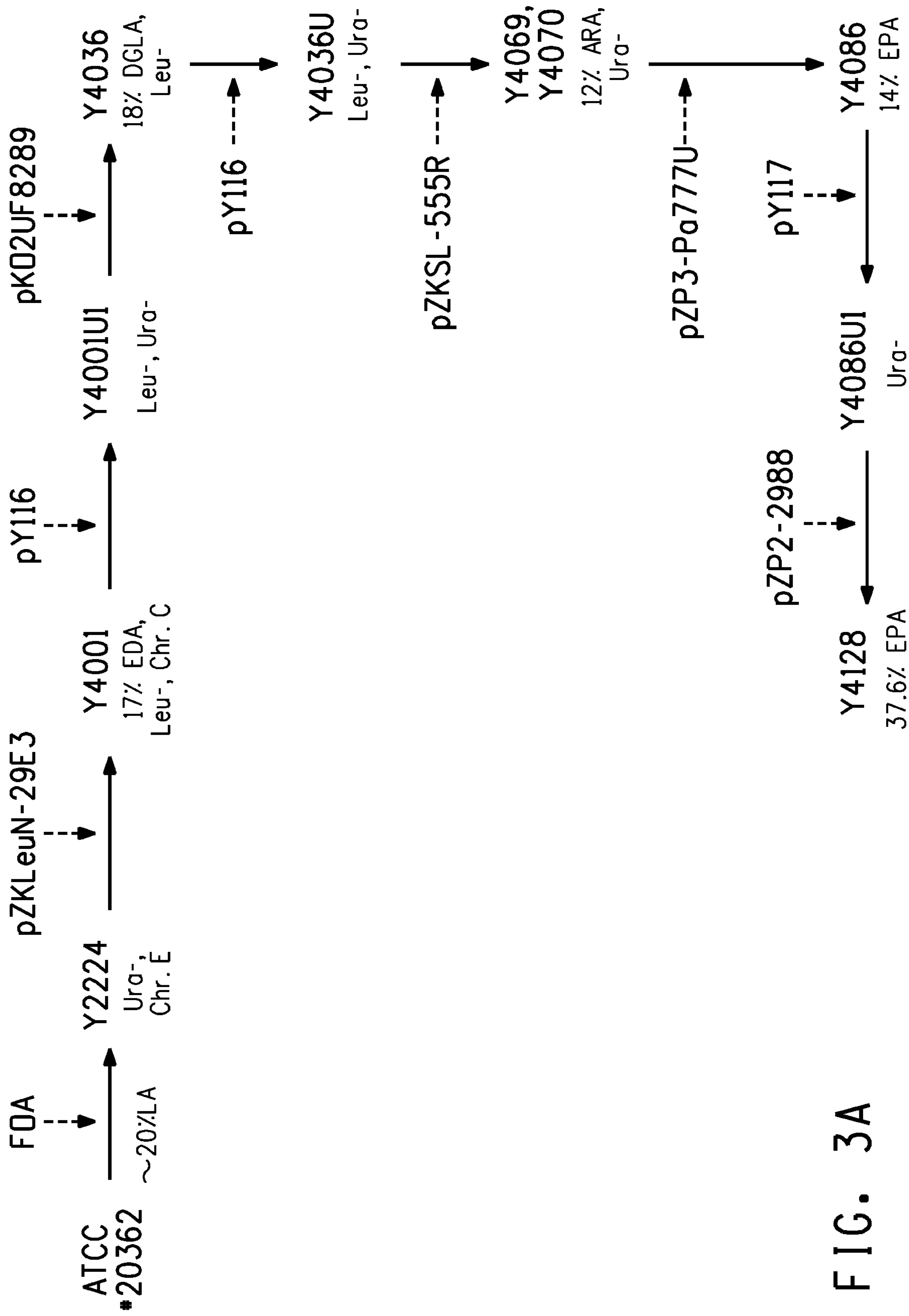


FIG. 3A

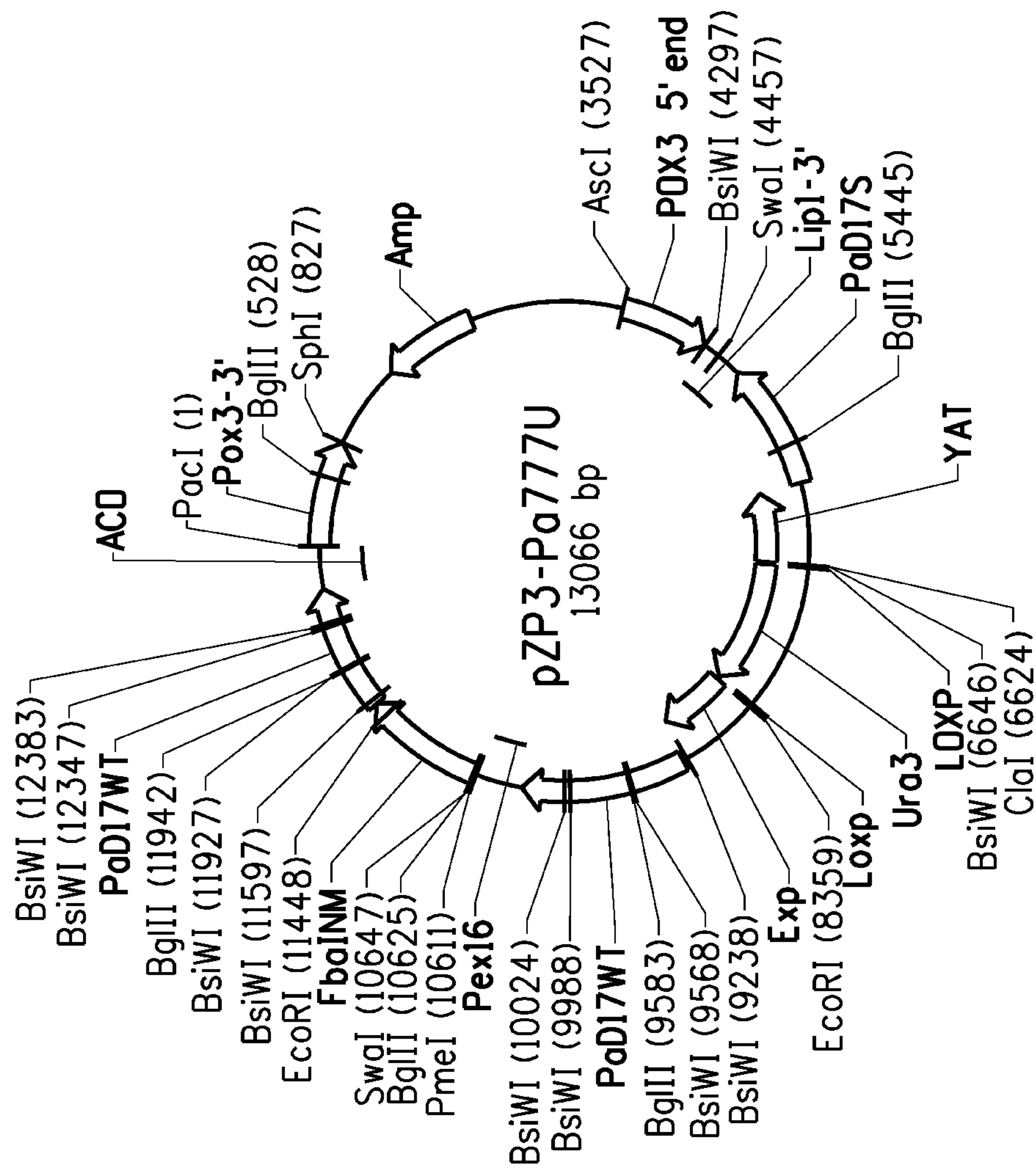


FIG. 3B

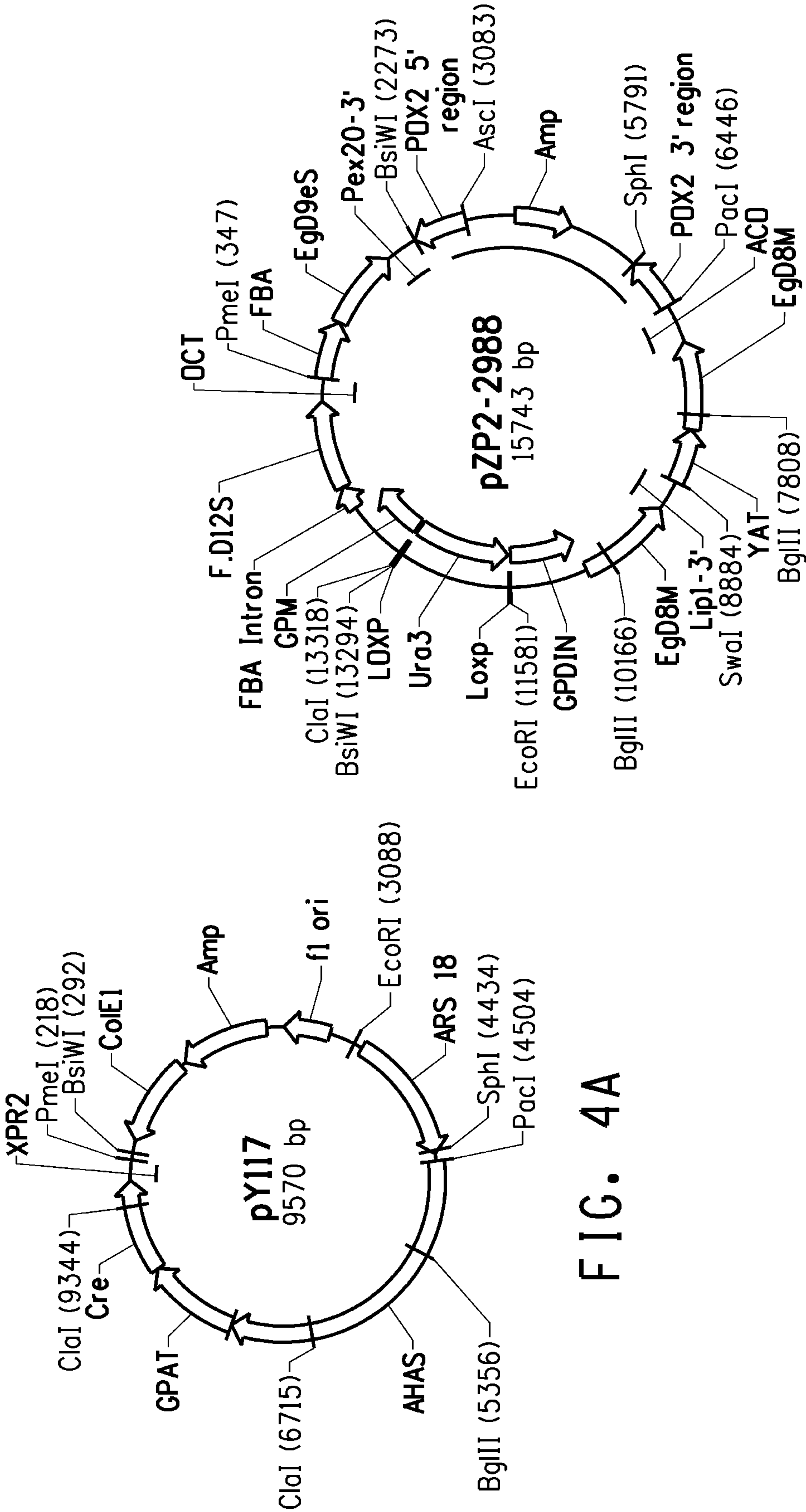


FIG. 4A

FIG. 4B

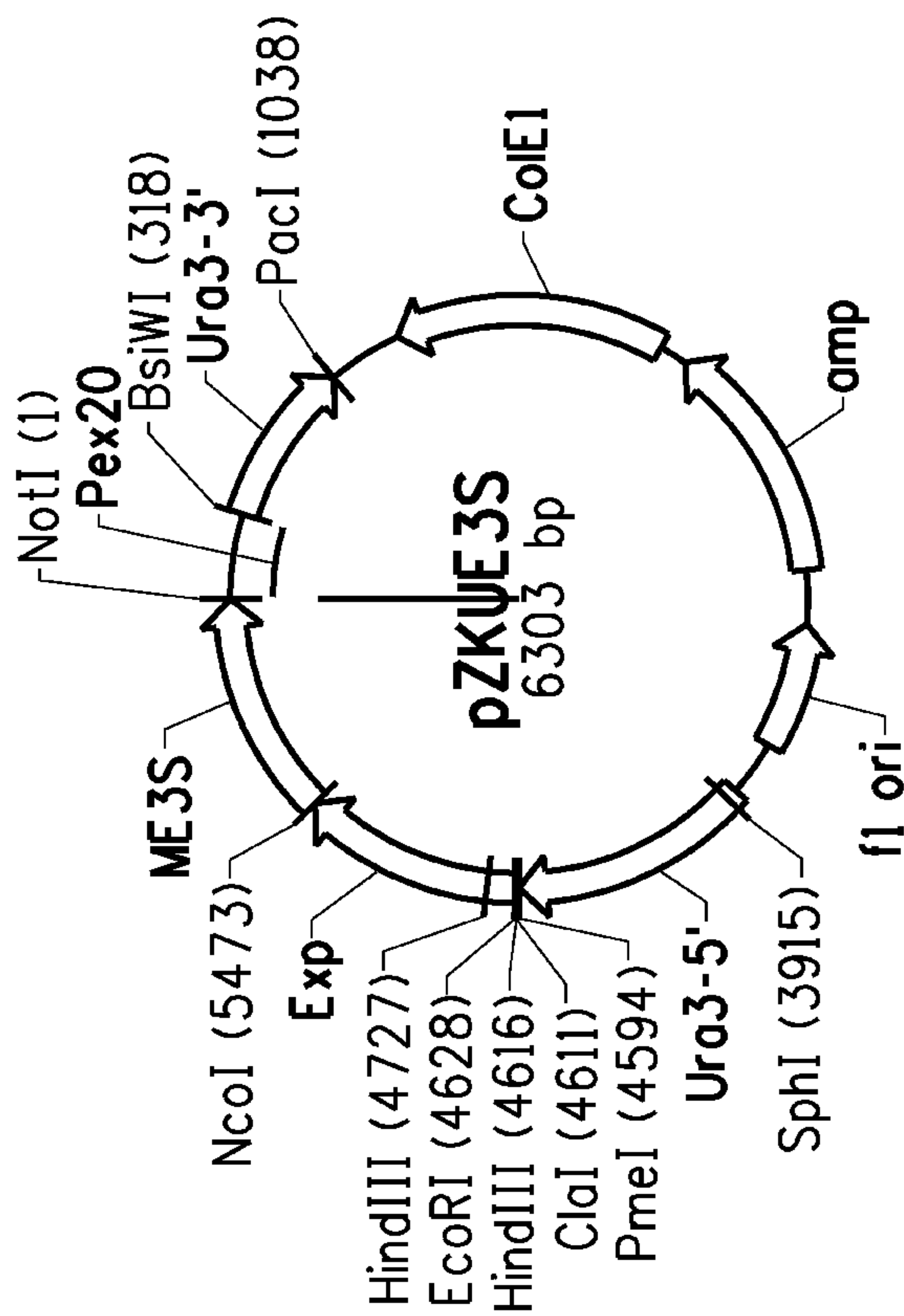


FIG. 5A

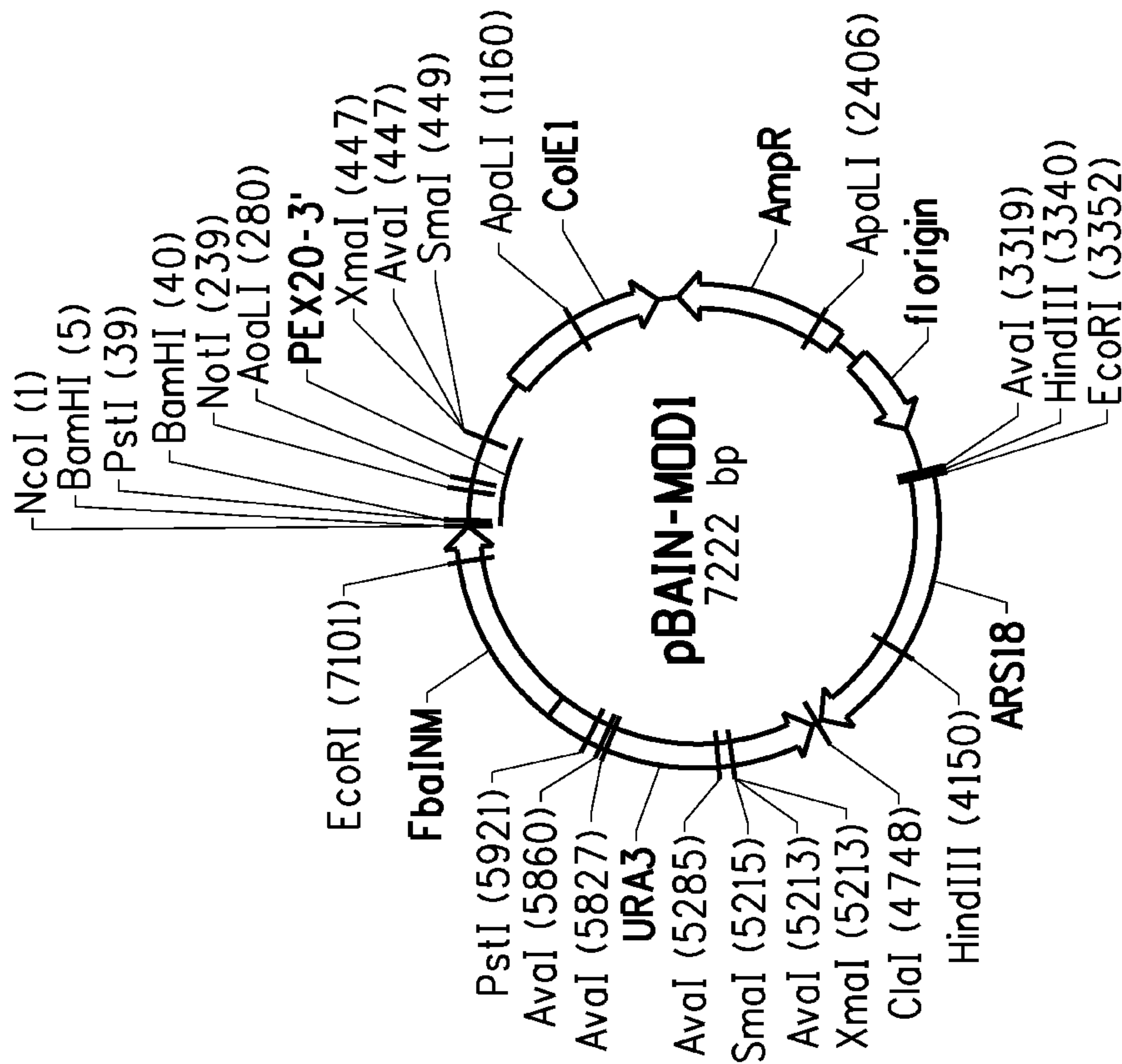


FIG. 5B

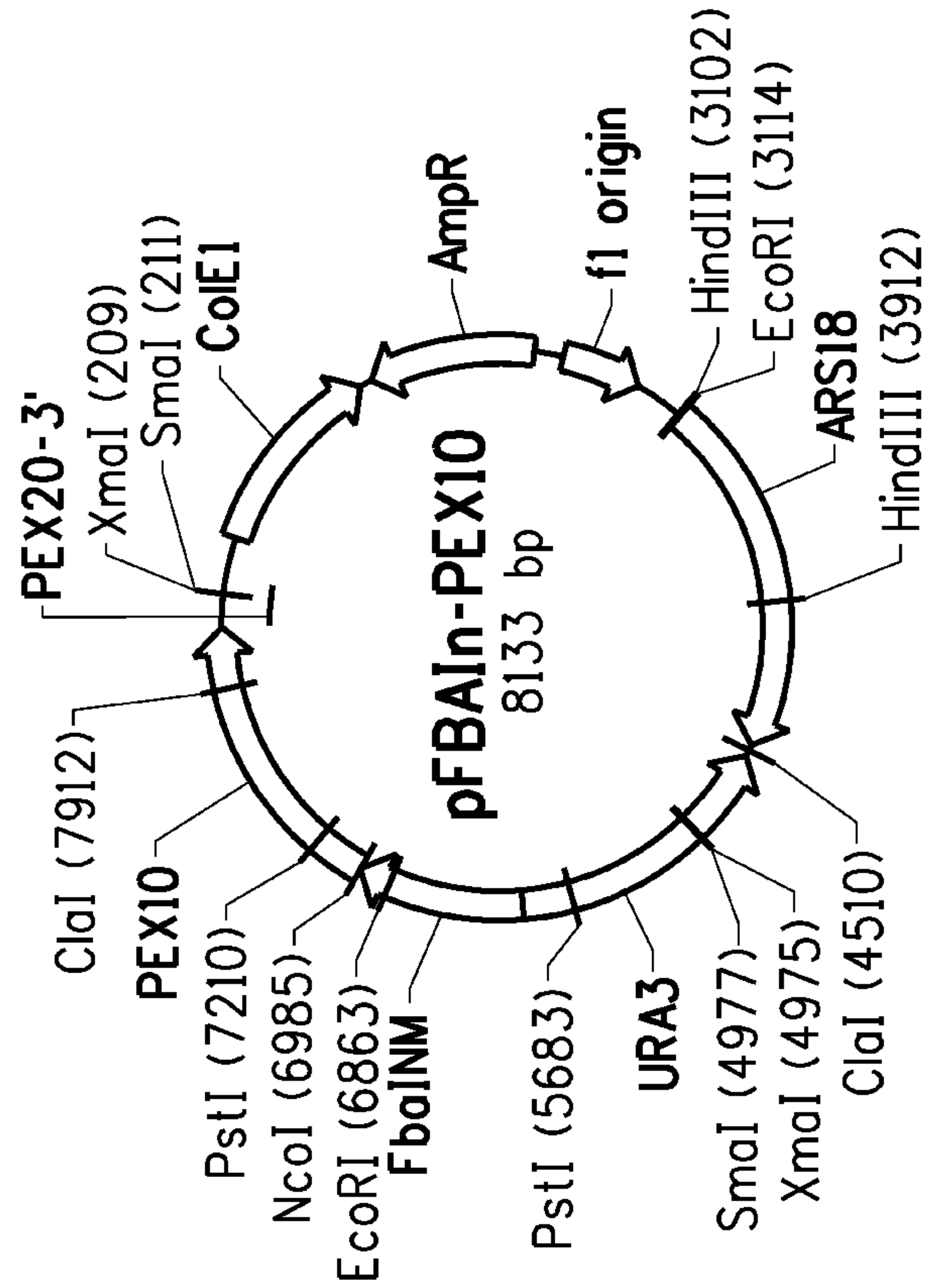


FIG. 6A

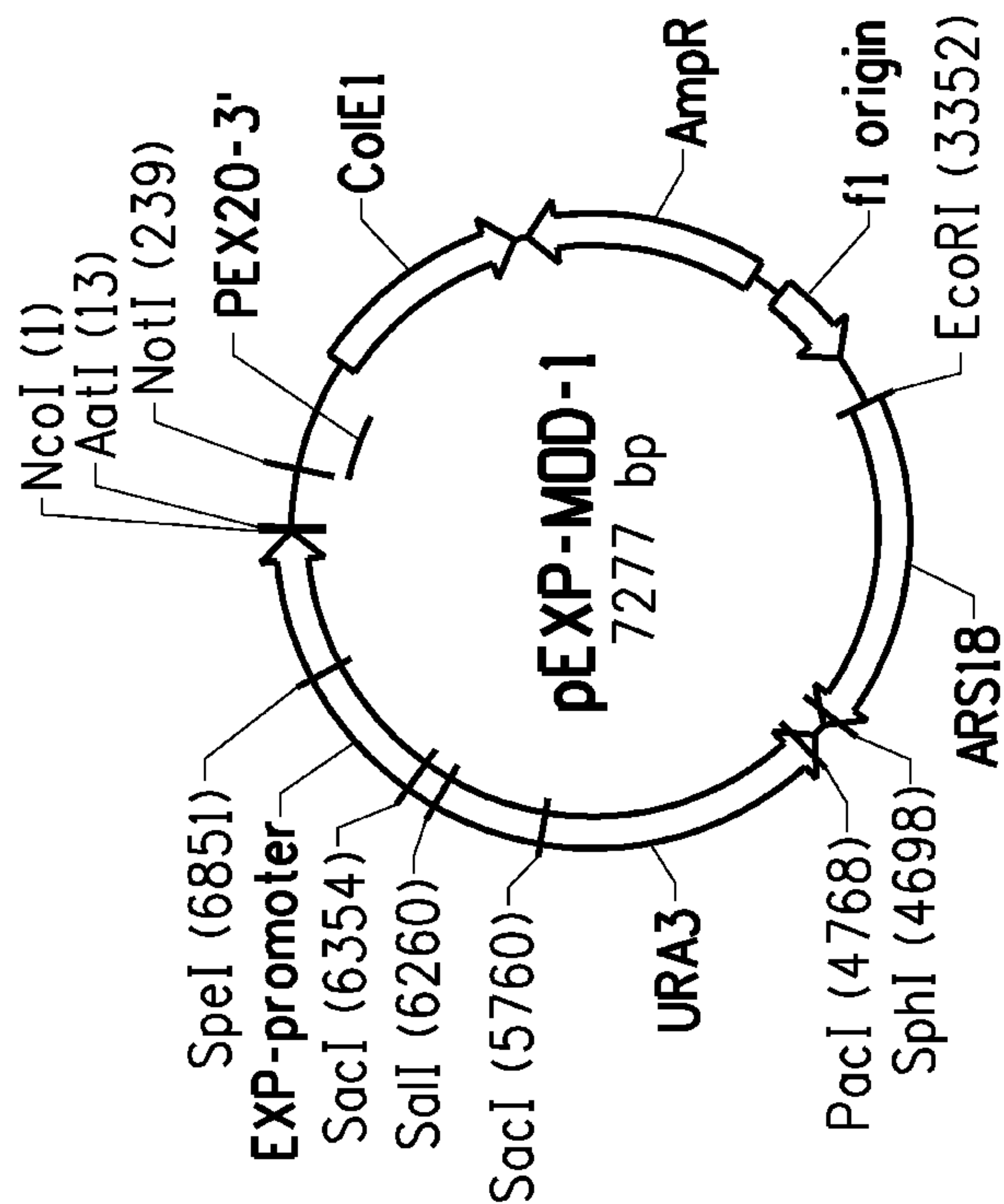


FIG. 6B

10/16

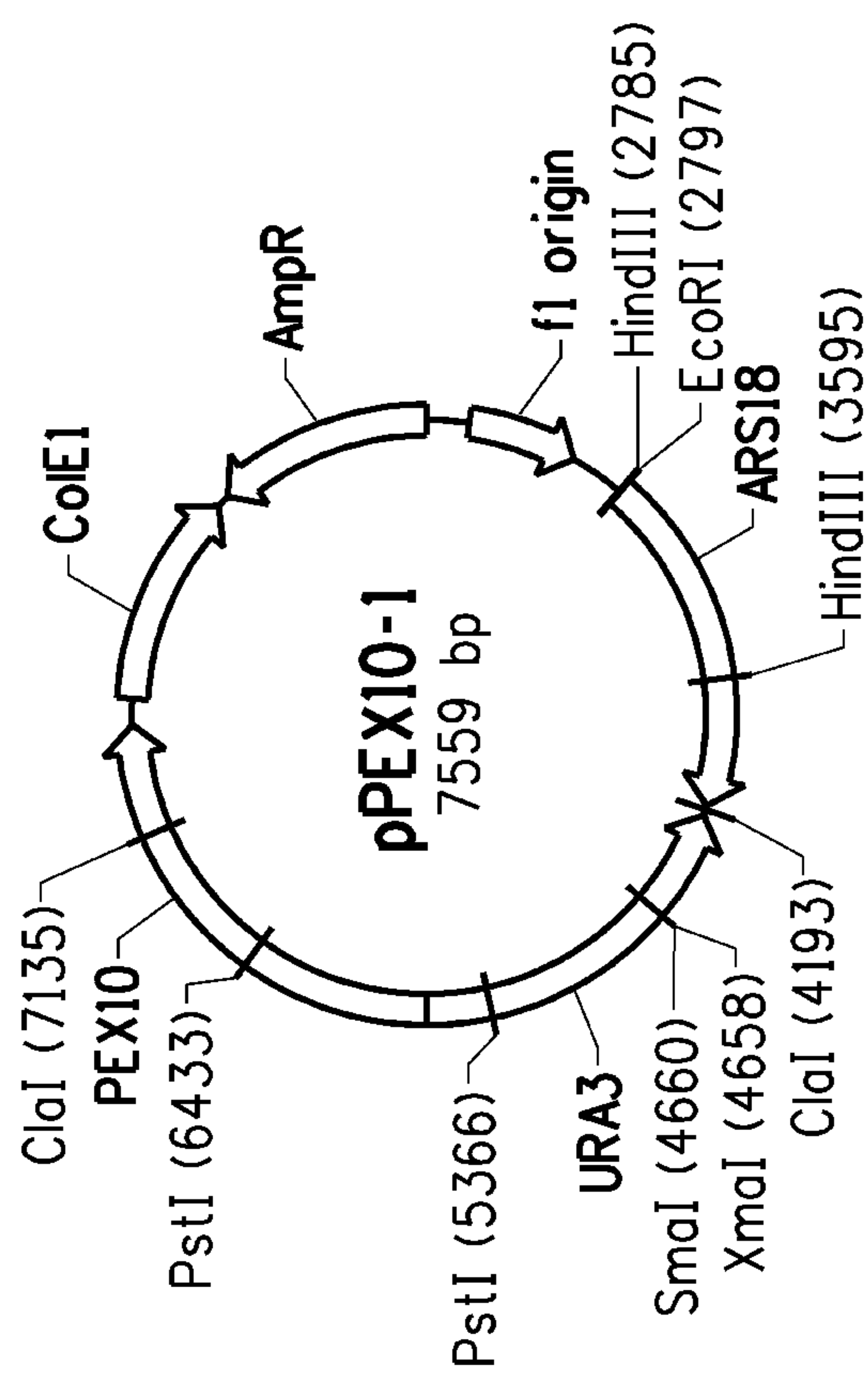


FIG. 7A

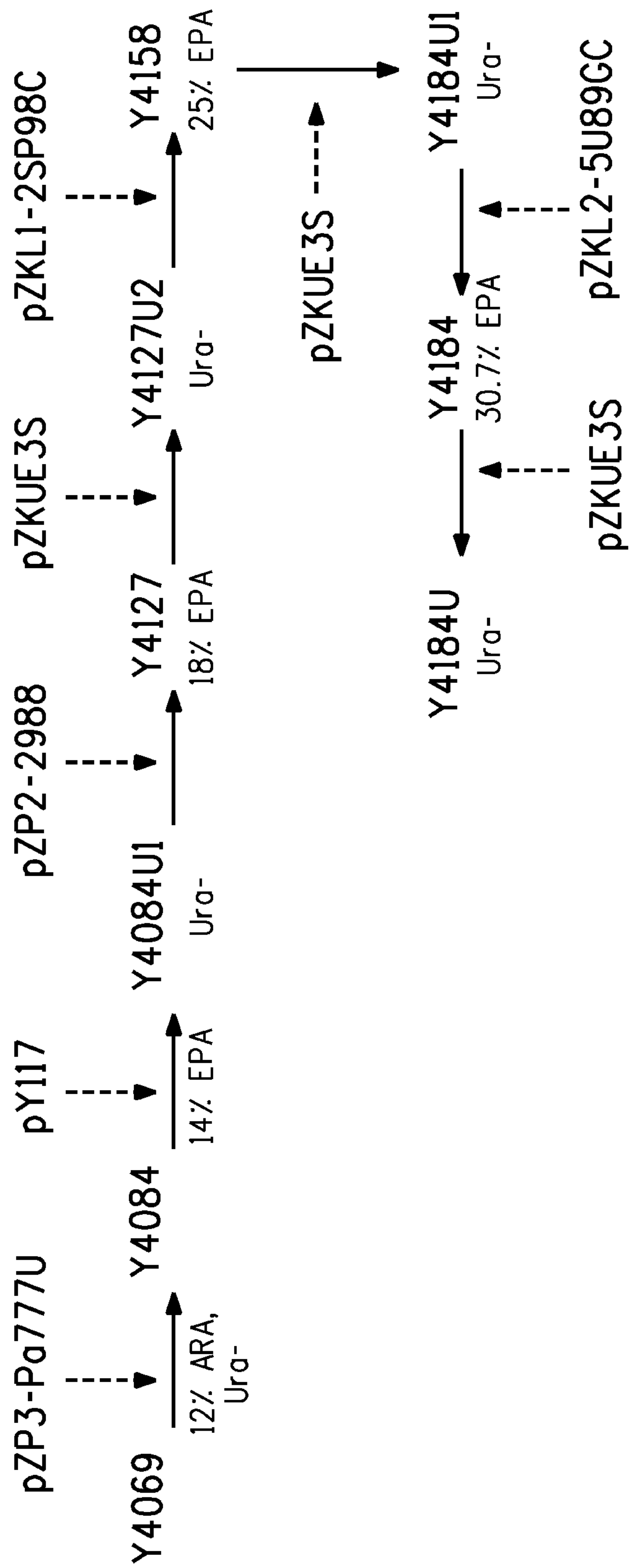


FIG. 7B

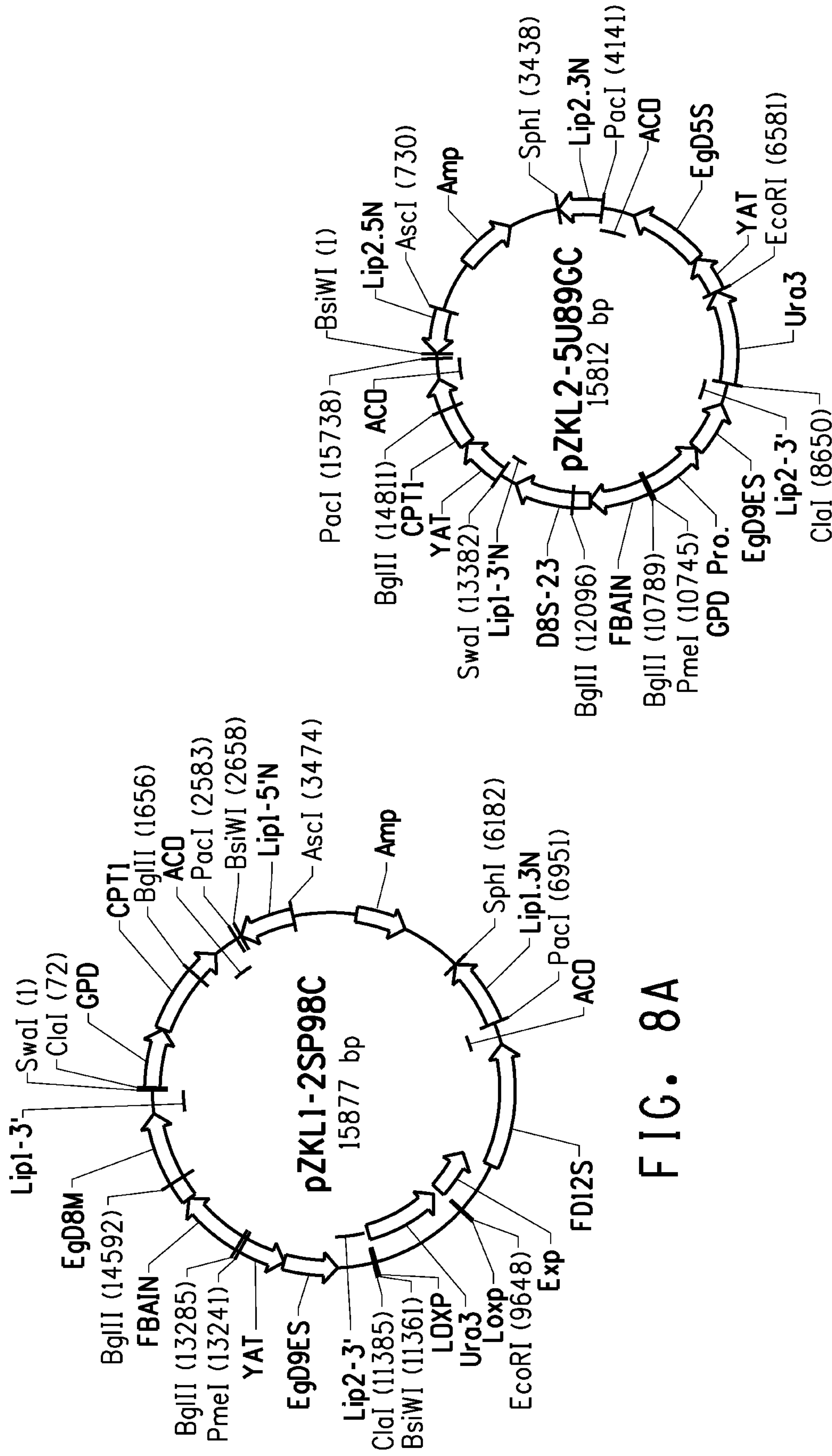


FIG. 8A

FIG. 8B

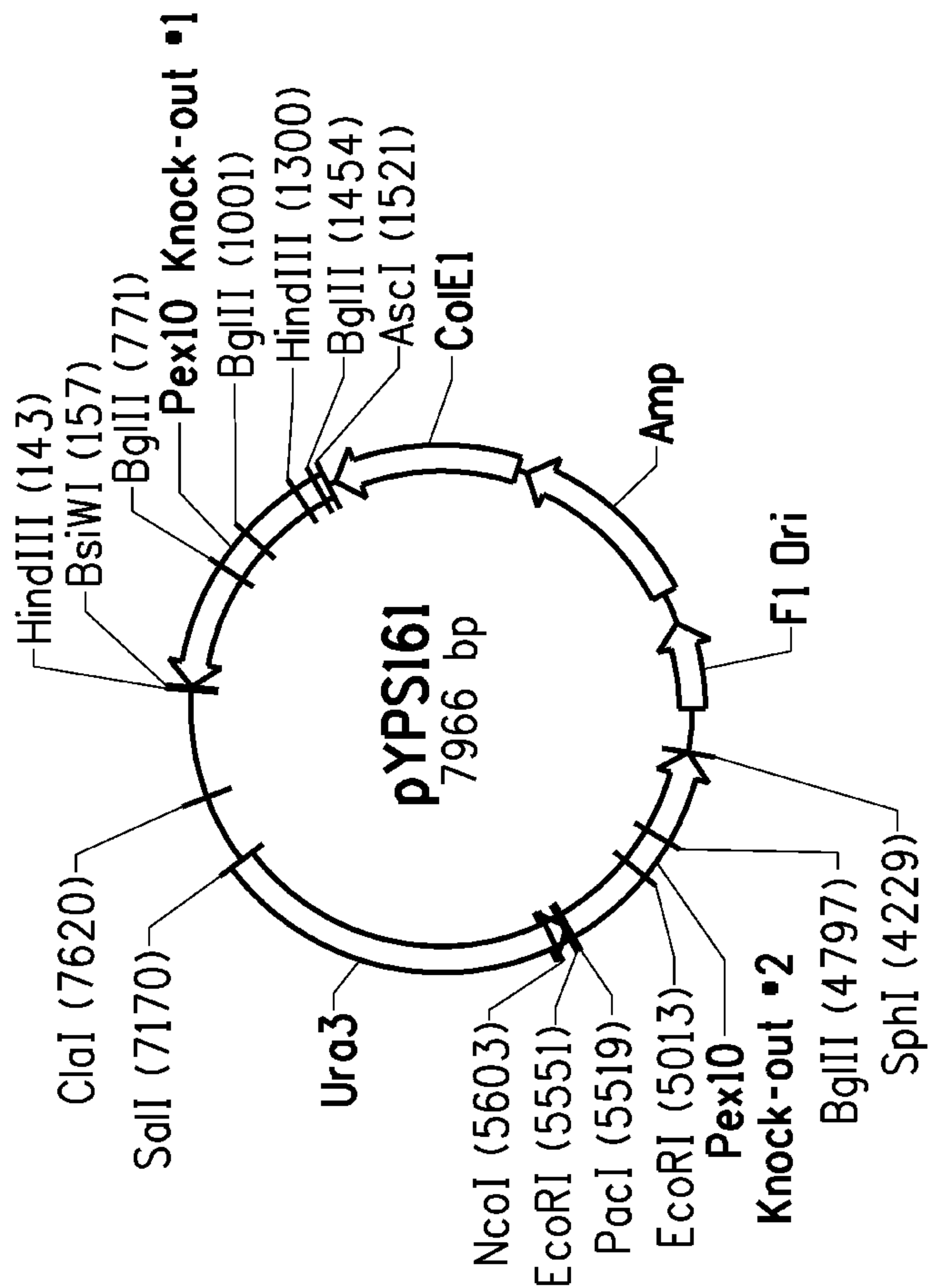


FIG. 9A

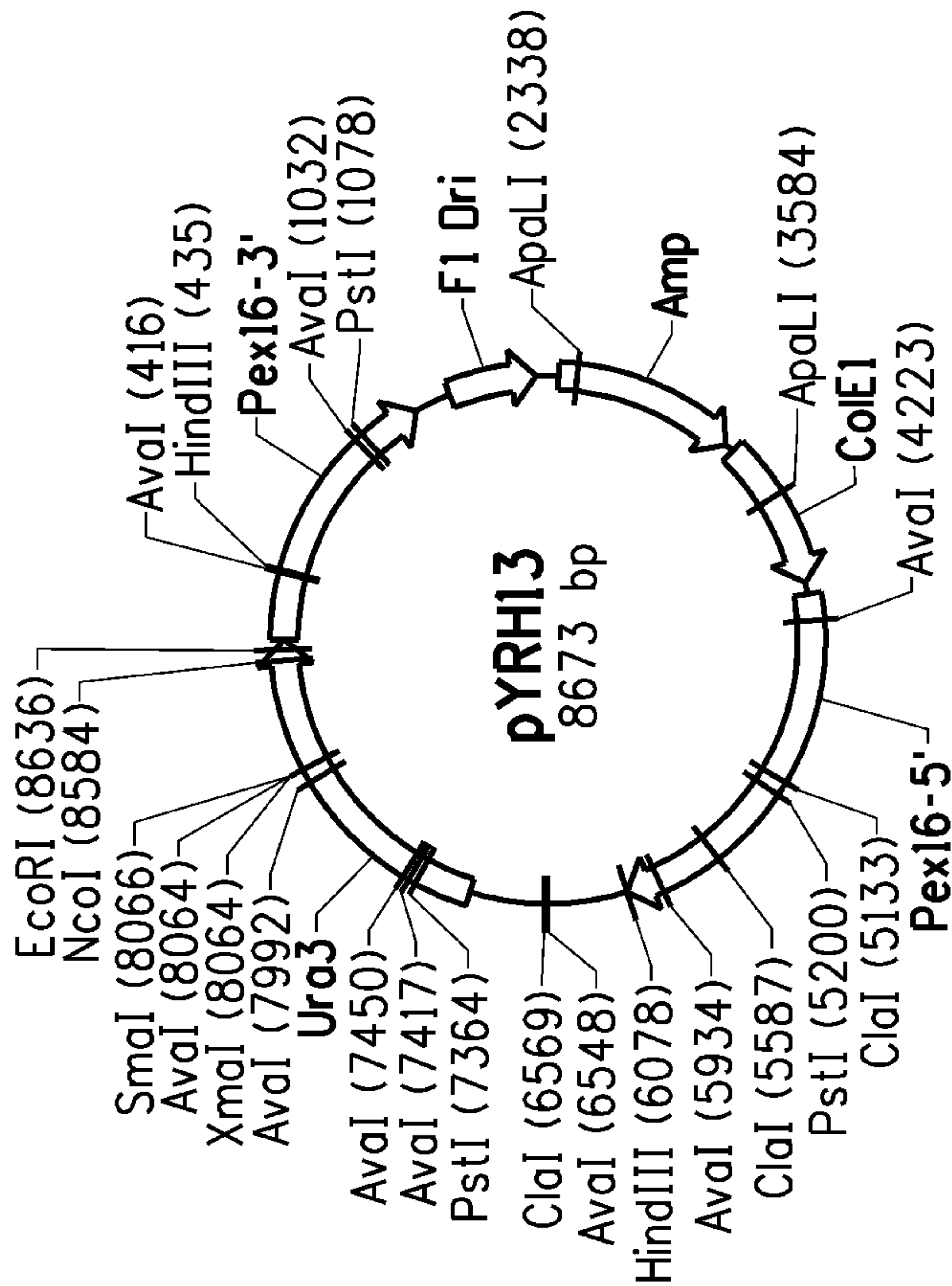


FIG. 9B

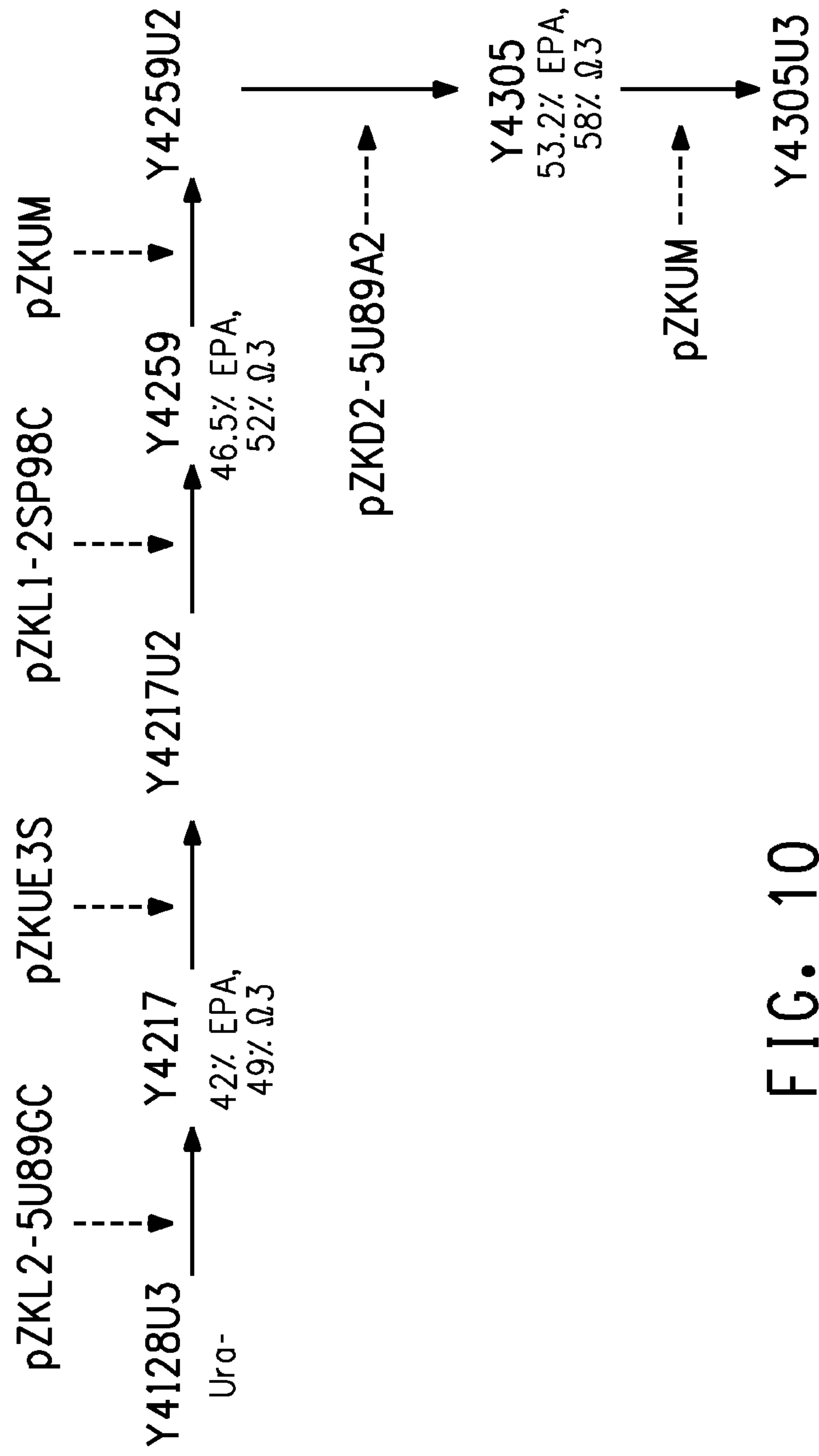


FIG. 10

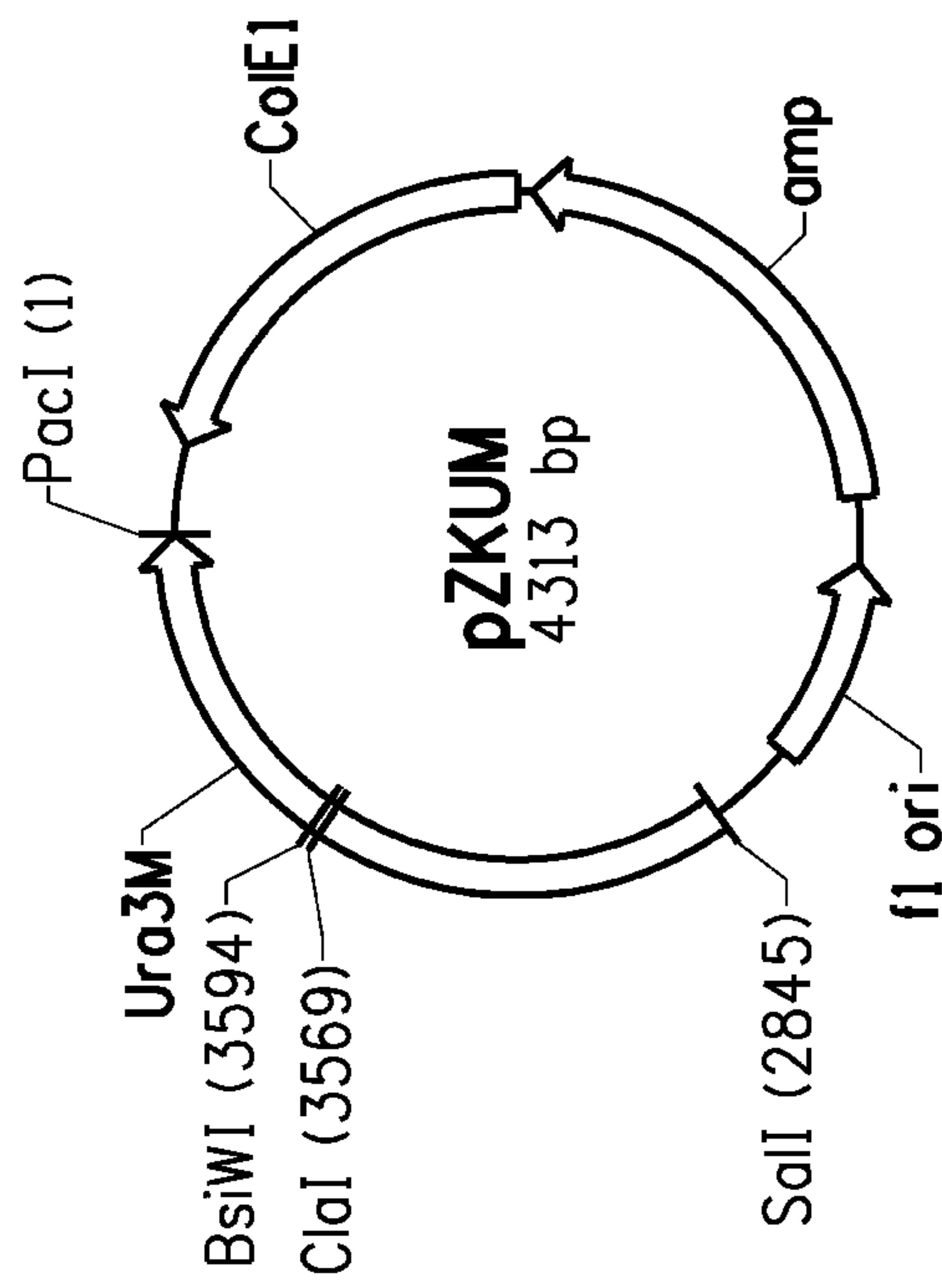


FIG. 11A

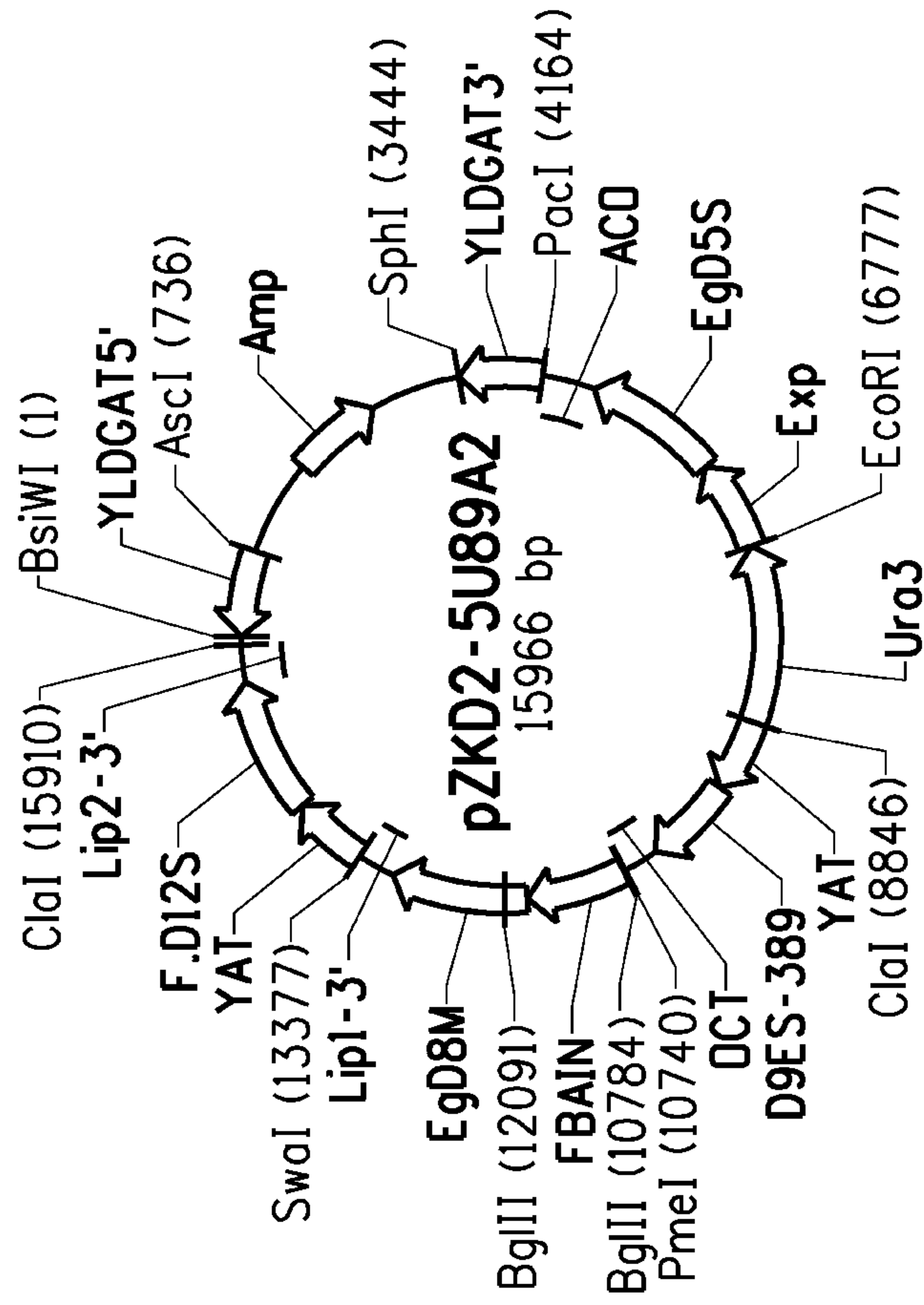


FIG. 11B

16/16

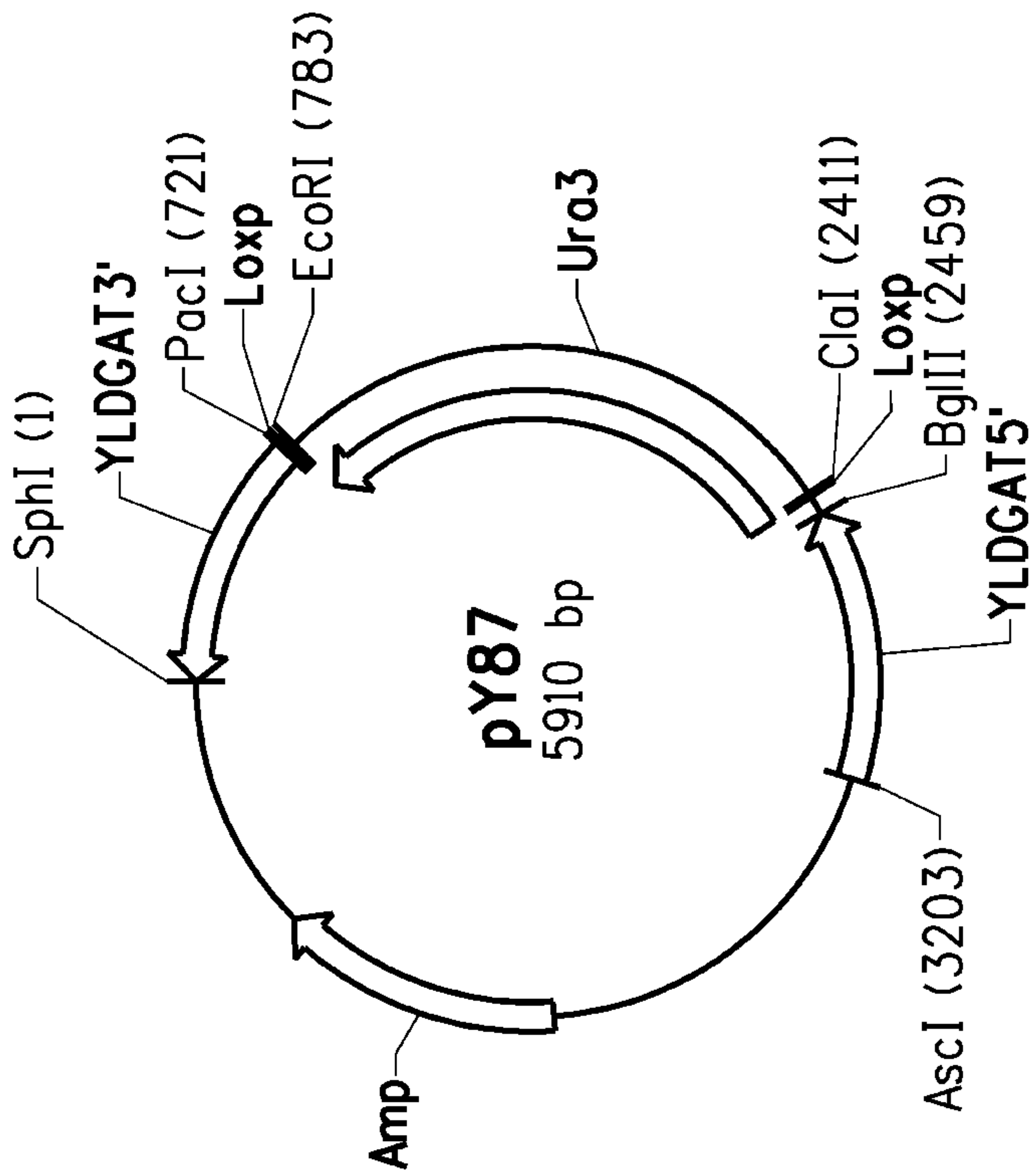


FIG. 12A

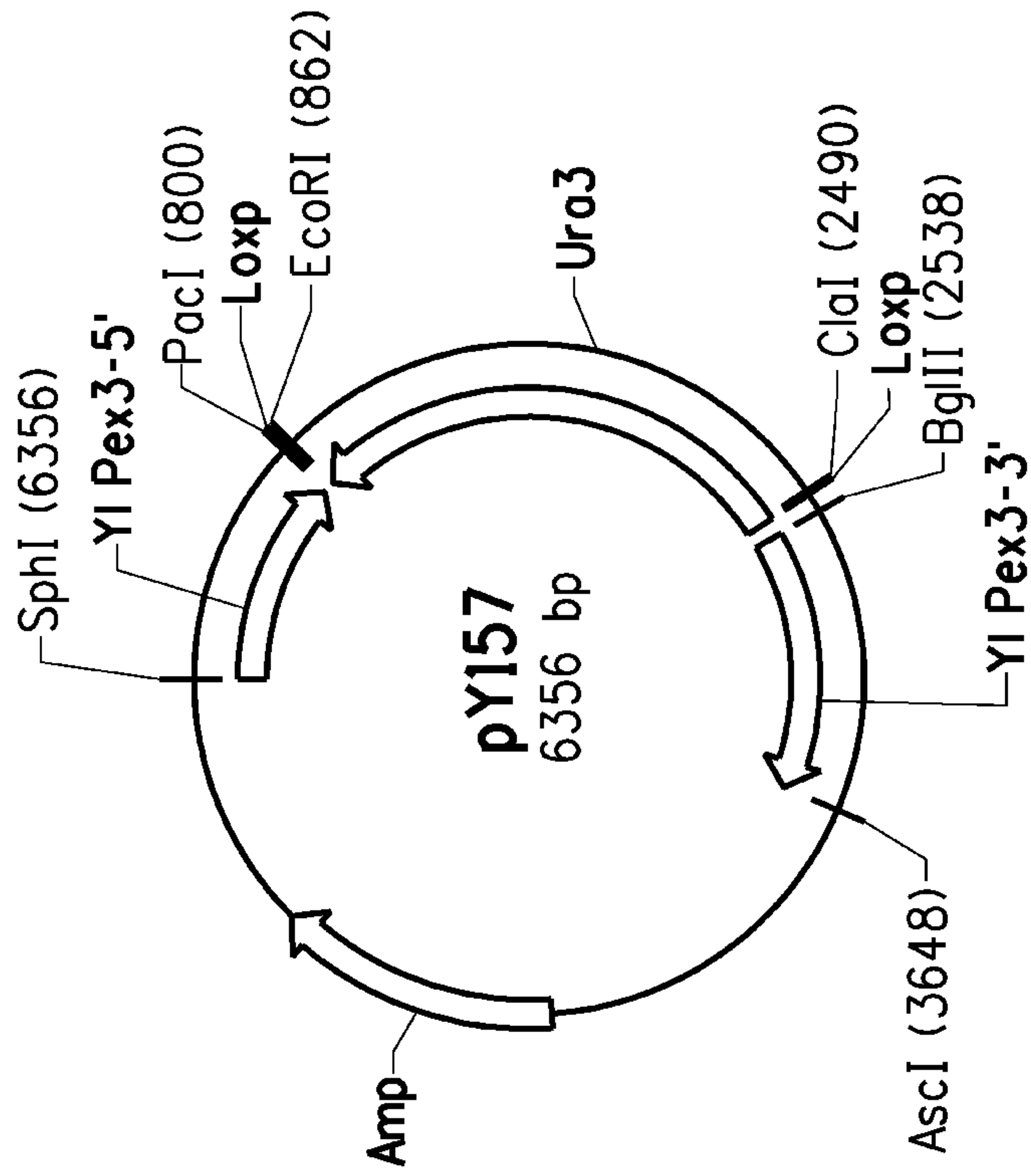


FIG. 12B

