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(54) Titre : PROMOTEURS DE LA GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE ET DE LA
PHOSPHOGLYCERATE MUTASE POUR L'EXPRESSION GENIQUE DANS DE LA LEVURE OLEAGINEUSE
(54) Title: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE MUTASE
PROMOTERS FOR GENE EXPRESSION IN OLEAGINOUS YEAST

(57) **Abrégé/Abstract:**

The promoter regions associated with the *Yarrowia lipolytica* glyceraldehyde-3-phosphate dehydrogenase (gpd) and phosphoglycerate mutase (gpm) genes have been found to be particularly effective for the expression of heterologous genes in oleaginous yeast. The promoter regions of the invention have been shown to drive high-level expression of genes involved in the production of ω -3 and ω -6 fatty acids.



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(54) Title: GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE MUTASE PROMOTERS FOR GENE EXPRESSION IN OLEAGINOUS YEAST

(57) Abstract: The promoter regions associated with the *Yarrowia lipolytica* glyceraldehyde-3-phosphate dehydrogenase (*gpd*) and phosphoglycerate mutase (*gpm*) genes have been found to be particularly effective for the expression of heterologous genes in oleaginous yeast. The promoter regions of the invention have been shown to drive high-level expression of genes involved in the production of ω -3 and ω -6 fatty acids.



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TITLEGLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND
PHOSPHOGLYCERATE MUTASE PROMOTERS FOR GENE
EXPRESSION IN OLEAGINOUS YEAST

5 This application claims the benefit of U.S. Provisional Application
No. 60/482263, filed June 25, 2003.

FIELD OF THE INVENTION

This invention is in the field of biotechnology. More specifically, this
invention pertains to promoter regions isolated from *Yarrowia lipolytica* that
10 are useful for gene expression in oleaginous yeast.

BACKGROUND OF THE INVENTION

Oleaginous yeast are defined as those organisms that are
naturally capable of oil synthesis and accumulation, wherein oil
accumulation ranges from at least about 25% up to about 80% of the
15 cellular dry weight. Genera typically identified as oleaginous yeast
include, but are not limited to: *Yarrowia*, *Candida*, *Rhodotorula*,
Rhodospiridium, *Cryptococcus*, *Trichosporon* and *Lipomyces*. More
specifically, illustrative oil-synthesizing yeast include: *Rhodospiridium*
toruloides, *Lipomyces starkeyii*, *L. lipoferus*, *Candida revkaufi*, *C.*
20 *pulcherrima*, *C. tropicalis*, *C. utilis*, *Trichosporon pullans*, *T. cutaneum*,
Rhodotorula glutinus, *R. graminis* and *Yarrowia lipolytica* (formerly
classified as *Candida lipolytica*).

The technology for growing oleaginous yeast with high oil content is
well developed (for example, see EP 0 005 277B1; Ratledge, C., *Prog.*
25 *Ind. Microbiol.* 16:119-206 (1982)). And, these organisms have been
commercially used for a variety of purposes in the past. For example,
various strains of *Yarrowia lipolytica* have historically been used for the
manufacture and production of: isocitrate lyase (DD259637); lipases
(SU1454852, WO2001083773, DD279267); polyhydroxyalkanoates
30 (WO2001088144); citric acid (RU2096461, RU2090611, DD285372,
DD285370, DD275480, DD227448, PL160027); erythritol (EP770683); 2-
oxoglutaric acid (DD267999); γ -decalactone (U.S. 6,451,565, FR2734843);
 γ -dodecalactone (EP578388); and pyruvic acid (JP09252790). Most
recently, however, the natural abilities of oleaginous yeast have been
35 enhanced by advances in genetic engineering, resulting in organisms
capable of producing polyunsaturated fatty acids ("PUFAs"). Specifically,
Picataggio et al. have demonstrated that *Yarrowia lipolytica* can be
engineered for production of ω -3 and ω -6 fatty acids, by introducing and

expressing genes encoding the ω -3/ ω -6 biosynthetic pathway (co-pending U.S. Patent Application No. 10/840579).

Recombinant production of any heterologous protein is generally accomplished by constructing an expression cassette in which the DNA
5 coding for the protein of interest is placed under the control of a promoter suitable for the host cell. The expression cassette is then introduced into the host cell (usually by plasmid-mediated transformation or targeted integration into the host genome) and production of the heterologous protein is achieved by culturing the transformed host cell under conditions
10 necessary for the proper function of the promoter contained within the expression cassette. Thus, the development of new host cells (e.g., oleaginous yeast) for recombinant production of proteins generally requires the availability of promoters that are suitable for controlling the expression of a protein of interest in the host cell.

15 A variety of strong promoters have been isolated from *Saccharomyces cerevisiae* that are useful for heterologous gene expression in yeast. For example, a glyceraldehyde-3-phosphate dehydrogenase (GPD) promoter was described by Bitter, G.A., and K.M. Egan (*Gene* 32(3):263-274 (1984)); and, a phosphoglycerate mutase
20 (GPM1) promoter was investigated by Rodicio, R. et al. (*Gene* 125(2): 125-133 (1993)). Several promoters have also been isolated from *Yarrowia lipolytica* that have been suitable for the recombinant expression of proteins. For example, U.S. 4,937,189 and EP220864 (Davidow et al.) disclose the sequence of the XPR2 gene (which encodes an inducible
25 alkaline extracellular protease) and upstream promoter region for use in expression of heterologous proteins. However, this promoter is only active at a pH above 6.0 on media lacking preferred carbon and nitrogen sources; and full induction requires high levels of peptone in the culture media. Subsequent analysis of the XPR2 promoter sequence by
30 Blanchin-Roland, S. et al. (EP832258; *Mol. Cell Biol.* 14(1):327-338 (1994)) determined that hybrid promoters containing only parts of the XPR2 promoter sequence may be used to obtain high level expression in *Yarrowia*, without the limitations resulting from use of the complete promoter sequence.

35 U.S. 6,265,185 (Muller et al.) describe yeast promoters from *Yarrowia lipolytica* for the translation elongation factor EF1- α (TEF) protein and ribosomal protein S7 that are suitable for expression cloning in yeast

and heterologous expression of proteins. These promoters were improved relative to the XPR2 promoter, when tested for yeast promoter activity on growth plates (Example 9, U.S. 6,265,185) and based on their activity in the pH range of 4-11.

5 Despite the utility of these known promoters, however, there is a need for new improved yeast promoters for metabolic engineering of yeast (oleaginous and non-oleaginous) and for controlling the expression of heterologous genes in yeast. Furthermore, possession of a suite of promoters that are regulatable under a variety of natural growth and
10 induction conditions in yeast will play an important role in industrial settings, wherein it is desirable to express heterologous polypeptides in commercial quantities in said hosts for economical production of those polypeptides. Thus, it is an object of the present invention to provide such promoters that will be useful for gene expression in a variety of yeast
15 cultures, and preferably in *Yarrowia sp.* cultures and other oleaginous yeast.

Applicants have solved the stated problem by identifying genes encoding a glyceraldehyde-3-phosphate dehydrogenase (GPD) and a phosphoglycerate mutase (GPM) from *Yarrowia lipolytica* and the
20 promoters responsible for driving expression of these native genes. Both promoters are useful for expression of heterologous genes in *Yarrowia* and have improved activity with respect to the TEF promoter.

SUMMARY OF THE INVENTION

The present invention provides methods for the expression of a
25 coding region of interest in a transformed yeast cell, using a promoter of the glyceraldehyde-3-phosphate dehydrogenase (*gpd*) or phosphoglycerate mutase (*gpm*) genes. Accordingly, the present invention provides a method for the expression of a coding region of interest in a transformed yeast cell comprising:

- 30 a) providing a transformed yeast cell having a chimeric gene comprising:
- (i) a promoter region of a *Yarrowia* gene selected from the group consisting of: a *gpm* gene and a *gpd* gene; and
 - (ii) a coding region of interest expressible in the yeast cell;
- 35 wherein the promoter region is operably linked to the coding region of interest; and
- b) growing the transformed yeast cell of step (a) under conditions

whereby the chimeric gene of step (a) is expressed.

In a preferred embodiment the invention provides a method for the production of an ω -3 or an ω -6 fatty acid comprising:

- a) providing a transformed oleaginous yeast comprising a chimeric
5 gene, comprising:
 - (i) a promoter region of a *Yarrowia* gene selected from the group consisting of: a *gpm* gene and a *gpd* gene; and
 - (ii) a coding region encoding at least one enzyme of the ω -3/ ω -6 fatty acid biosynthetic pathway;
- 10 wherein the promoter region and coding region are operably linked; and
- (b) contacting the transformed oleaginous yeast of step (a) under conditions whereby the at least one enzyme of the ω -3/ ω -6 fatty biosynthetic pathway is expressed and a ω -3 or ω -6
15 fatty acid is produced; and
- (c) optionally recovering the ω -3 or ω -6 fatty acid.

Additionally the invention provides an isolated nucleic acid molecule comprising a *gpd* promoter selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24 and SEQ ID NO:43.

- 20 In similar fashion the invention provides an isolated nucleic acid molecule comprising a *gpm* promoter selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:44.

BRIEF DESCRIPTION OF THE DRAWINGS AND SEQUENCE DESCRIPTIONS

- 25 Figure 1A and 1B shows an alignment of known glyceraldehyde-3-phosphate dehydrogenase (GPD) proteins from *Saccharomyces cerevisiae* (GenBank Accession No. CAA24607), *Schizosaccharomyces pombe* (GenBank Accession No. NP_595236), *Aspergillus oryzae* (GenBank Accession No. AAK08065), *Paralichthys olivaceus* (GenBank
30 Accession No. BAA88638), *Xenopus laevis* (GenBank Accession No. P51469) and *Gallus gallus* (GenBank Accession No. DECHG3), used to identify two conserved regions within the sequence alignment.

Figure 2 shows an alignment of amino acids encoding portions of the GPD protein from *Yarrowia lipolytica*, *Schizosaccharomyces pombe*, *Gallus gallus* and *Xenopus laevis*.

Figure 3 shows an alignment of phosphoglycerate mutase (GPM) proteins from *Yarrowia lipolytica* and *Saccharomyces cerevisiae*.

Figure 4 graphically represents the relationship between SEQ ID NOs:11, 12, 23-26 and 43, each of which relates to glyceraldehyde-3-phosphate dehydrogenase (GPD) in *Yarrowia lipolytica*.

Figure 5 graphically represents the relationship between SEQ ID NOs:14-16, 27, 28 and 44, each of which relates to phosphoglycerate mutase (GPM) in *Yarrowia lipolytica*.

Figure 6 illustrates the construction of plasmid vector pY5-4.

Figures 7A, 7B, 7C and 7D provide plasmid maps for pY5-10, pY5-30, pYZGDG and pYZGMG, respectively.

Figure 8A is an image of a cell culture comparing the promoter activity of TEF and GPD in *Yarrowia lipolytica* as determined by histochemical staining. Figure 8B is an image of a cell culture comparing the promoter activity of TEF and GPM in *Y. lipolytica* as determined by histochemical staining.

Figure 9A is a graph comparing the promoter activity of TEF and GPD as determined fluorometrically. Figure 9B is a graph comparing the promoter activity of TEF and GPM as determined fluorometrically.

Figure 10 illustrates the ω -3/ ω -6 fatty acid biosynthetic pathway.

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

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

SEQ ID NOs:1-6 correspond to the GPD amino acid sequences of *Saccharomyces cerevisiae* (GenBank Accession No. CAA24607),

Schizosaccharomyces pombe (GenBank Accession No. NP_595236),
Aspergillus oryzae (GenBank Accession No. AAK08065), *Paralichthys*
olivaceus (GenBank Accession No. BAA88638), *Xenopus laevis*
 (GenBank Accession No. P51469) and *Gallus gallus* (GenBank Accession
 5 No. DECHG3), respectively.

SEQ ID NOs:7 and 8 correspond to conserved amino acid regions
 of the GPD protein.

SEQ ID NOs:9 and 10 correspond to the degenerate primers YL193
 and YL194, respectively, used for isolating an internal portion of the
 10 *Yarrowia lipolytica* GPD gene.

SEQ ID NO:11 encodes a 507 bp internal portion of the *Yarrowia*
lipolytica GPD gene, while SEQ ID NO:12 is the corresponding amino acid
 sequence.

SEQ ID NO:13 corresponds to the *Saccharomyces cerevisiae* GPM
 15 protein (GenBank Accession No. NP_012770).

SEQ ID NO:14 corresponds to Contig 2217, comprising the
 complete nucleotide coding sequence for the *Yarrowia lipolytica* GPM
 protein.

SEQ ID NO:15 corresponds to the deduced nucleotide sequence of
 20 the *Yarrowia lipolytica* GPM coding region, while SEQ ID NO:16
 corresponds to the amino acid sequence.

SEQ ID NOs:17-22 correspond to primers YL206, YL196, YL207,
 YL197, YL208 and YL198, respectively, used for genome walking.

SEQ ID NO:23 corresponds to a 1848 bp fragment designated as
 25 "GPDP", comprising 1525 bp upstream of the GPD gene and an additional
 323 bp representing a 5' portion of the GPD gene in *Yarrowia lipolytica*.

SEQ ID NO:24 corresponds to an assembled 2316 bp contig of
 DNA, corresponding to the -1525 to +791 region of the GPD gene,
 wherein the 'A' position of the 'ATG' translation initiation codon is
 30 designated as +1.

SEQ ID NO:25 corresponds to a partial cDNA sequence encoding
 the *Yarrowia lipolytica* GPD gene, while SEQ ID NO:26 is the
 corresponding amino acid sequence.

SEQ ID NO:27 corresponds to a 953 bp fragment designated as
 35 "GPML", corresponding to the -875 to +78 region of the GPM gene,
 wherein the 'A' position of the 'ATG' translation initiation codon is
 designated as +1.

SEQ ID NO:28 corresponds to an assembled 1537 bp contig of DNA, corresponding to the -875 to +662 region of the GPM gene, wherein the 'A' position of the 'ATG' translation initiation codon is designated as +1.

5 SEQ ID NOs:29 and 30 correspond to primers YL33 and YL34, respectively, used for amplifying the reporter gene GUS.

SEQ ID NOs:31 and 32 correspond to primers TEF5' and TEF3', respectively, used to isolate the TEF promoter.

10 SEQ ID NOs:33 and 34 correspond to primers XPR5' and XPR3', respectively, used to isolate the XPR2 transcriptional terminator.

SEQ ID NOs:35-42 correspond to primers YL1, YL2, YL3, YL4, YL23, YL24, YL9 and YL10, respectively, used for site-directed mutagenesis during construction of the pY5-10 plasmid.

15 SEQ ID NO:43 corresponds to a 971 bp fragment designated as "GPDPPro", and identified herein as the putative GPD promoter in *Yarrowia lipolytica*. This fragment corresponds to the -968 to +3 region of the GPD gene, wherein the 'A' position of the 'ATG' translation initiation codon is designated as +1.

20 SEQ ID NO:44 corresponds to a 878 bp fragment designated as "GPMLPro", and identified herein as the putative GPM promoter in *Yarrowia lipolytica*. This fragment corresponds to the -875 to +3 region of the GPM gene, wherein the 'A' position of the 'ATG' translation initiation codon is designated as +1.

25 SEQ ID NOs:45 and 46 correspond to primers YL211 and YL212, respectively, used to amplify the putative GPD promoter.

SEQ ID NOs:47 and 48 correspond to primers YL203 and YL204, respectively, used to amplify the putative GPM promoter.

SEQ ID NOs:49-54 correspond to primers YL5, YL6, YL7, YL8, YL61 and YL62, respectively, used for construction of plasmid pY5-13.

30 SEQ ID NOs:55 and 56 correspond to primers GPDsense and GPDantisense, respectively, used to amplify GPDPPro.

SEQ ID NO:57 corresponds to the nucleotide sequence of the *Fusarium moniliforme* strain M-8114 Δ 15 desaturase coding region, while SEQ ID NO:58 corresponds to the amino acid sequence.

35 SEQ ID NOs:59 and 60 correspond to primers P192 and P193, respectively, used to amplify the *F.moniliforme* Δ 15 desaturase.

DETAILED DESCRIPTION OF THE INVENTION

In accordance with the subject invention, Applicants describe the isolation and characterization of promoters and genes from an oleaginous yeast, *Yarrowia lipolytica*. These promoter regions, isolated upstream of
5 the glyceraldehyde-3-phosphate dehydrogenase (GPD) and phosphoglycerate mutase (GPM) genes, are useful for genetic engineering in *Y. lipolytica* and other yeast for the production of heterologous polypeptides.

Preferred heterologous polypeptides of the present invention are
10 those that are involved in the synthesis of microbial oils and particularly polyunsaturated fatty acids (PUFAs). PUFAs, or derivatives thereof, made by the methodology disclosed herein can be used in many applications. For example, the PUFAs can be used as dietary substitutes, or supplements, particularly infant formulas, for patients undergoing
15 intravenous feeding or for preventing or treating malnutrition. Alternatively, the purified PUFAs (or derivatives thereof) may be incorporated into cooking oils, fats or margarines formulated so that in normal use the recipient would receive the desired amount for dietary supplementation. The PUFAs may also be incorporated into infant formulas, nutritional
20 supplements or other food products, and may find use as anti-inflammatory or cholesterol lowering agents. Optionally, the compositions may be used for pharmaceutical use (human or veterinary). In this case, the PUFAs are generally administered orally but can be administered by any route by which they may be successfully absorbed, e.g., parenterally
25 (e.g., subcutaneously, intramuscularly or intravenously), rectally, vaginally or topically (e.g., as a skin ointment or lotion).

Thus, the present invention advances the art by providing methods for the expression of a coding region of interest in a transformed yeast comprising: a) providing a transformed yeast cell having a chimeric gene
30 comprising (i) a promoter region of a *gpd* gene or *gpm* gene; and (ii) a coding region of interest expressible in the host cell, wherein the promoter region is operably linked to the coding region of interest; and b) growing the transformed yeast cell of step (a) in the presence of a fermentable carbon source, wherein the chimeric gene is expressed and optionally
35 isolated from the cultivation medium. In preferred embodiments, the promoter region comprises a sequence selected from the group consisting of SEQ ID NOs: 23, 24, 27, 28, 43 and 44.

Definitions

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

"Glyceraldehyde-3-phosphate dehydrogenase" is abbreviated GPD.

5 "Phosphoglycerate mutase" is abbreviated GPM.

"Open reading frame" is abbreviated ORF.

"Polymerase chain reaction" is abbreviated PCR.

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

The term "oleaginous" refers to those organisms that tend to store
10 their energy source in the form of lipid (Weete, In: Fungal Lipid
Biochemistry, 2nd Ed., Plenum, 1980). Generally, the cellular PUFA
content of these 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 gradually decreases
15 during the late stationary and death phases (Yongmanitchai and Ward,
Appl. Environ. Microbiol. 57:419-25 (1991)).

The term "oleaginous yeast" refers to those microorganisms
classified as yeast that can accumulate at least 25% of their dry cell weight
as oil. Examples of oleaginous yeast include (but are no means limited to)
20 the following genera: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*,
Cryptococcus, *Trichosporon* and *Lipomyces*.

The term "fermentable carbon source" will refer to a carbon source
that a microorganism will metabolize to derive energy. Typical carbon
sources for use in the present invention include, but are not limited to:
25 monosaccharides, oligosaccharides, polysaccharides, alkanes, fatty acids,
esters of fatty acids, monoglycerides, diglycerides, triglycerides, carbon
dioxide, methanol, formaldehyde, formate and carbon-containing amines.

The term "GPD" refers to a glyceraldehyde-3-phosphate
dehydrogenase enzyme (E.C. 1.2.1.12) encoded by the *gpd* gene and
30 which converts D-glyceraldehyde 3-phosphate to 3-phospho-D-glyceroyl
phosphate during glycolysis. The partial coding region of a
representative *gpd* gene isolated from *Yarrowia lipolytica* is provided as
SEQ ID NOs:25 and 26; specifically, the sequence lacks ~115 amino acids
that encode the C-terminus of the gene (based on alignment with other
35 known *gpd* sequences).

The term "GPD promoter" or "GPD promoter region" refers to the 5'
upstream untranslated region in front of the 'ATG' translation initiation

codon of GPD and that is necessary for expression. Examples of suitable GPD promoter regions are provided as SEQ ID NOs:23 and 43, but these are not intended to be limiting in nature.

5 The term "GPM" refers to a phosphoglycerate mutase enzyme (EC 5.4.2.1) encoded by the *gpm* gene and which is responsible for the interconversion of 3-phosphoglycerate and 2-phosphoglycerate during glycolysis. A representative *gpm* gene from *Saccharomyces cerevisiae* is GenBank Accession No. NP_012770 (SEQ ID NO:13); a *gpm* gene isolated from *Yarrowia lipolytica* is provided as SEQ ID NO:15.

10 The term "GPM promoter" or "GPM promoter region" refers to the 5' upstream untranslated region in front of the 'ATG' translation initiation codon of GPM and that is necessary for expression. Examples of suitable GPM promoter regions are provided as SEQ ID NOs:27 and 44, but these are not intended to be limiting in nature.

15 The term "promoter activity" will refer to an assessment of the transcriptional efficiency of a promoter. This may, for instance, be determined directly by measurement of the amount of mRNA transcription from the promoter (e.g., by Northern blotting or primer extension methods) or indirectly by measuring the amount of gene product expressed from the promoter.

20 As used herein, an "isolated nucleic acid molecule" is a polymer of RNA or DNA that is single- or double-stranded, optionally containing synthetic, non-natural or altered nucleotide bases. An isolated nucleic acid molecule in the form of a polymer of DNA may be comprised of one or more segments of cDNA, genomic DNA or synthetic DNA.

25 A nucleic acid molecule is "hybridizable" to another nucleic acid molecule, such as a cDNA, genomic DNA, or RNA molecule, when a single-stranded form of the nucleic acid molecule can anneal to the other nucleic acid molecule 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), particularly Chapter 11 and Table 11.1 therein (entirely incorporated herein by reference). The conditions of temperature and ionic strength determine the "stringency" of the hybridization. Stringency conditions can be adjusted to screen for

moderately similar fragments (such as homologous sequences from distantly related organisms), to highly similar fragments (such as genes that duplicate functional enzymes from closely related organisms).

Post-hybridization washes determine stringency conditions. One set of preferred conditions uses a series of washes starting with 6X SSC, 0.5% SDS at room temperature for 15 min, then repeated with 2X SSC, 0.5% SDS at 45°C for 30 min, and then repeated twice with 0.2X SSC, 0.5% SDS at 50°C for 30 min. A more preferred set of stringent conditions uses higher temperatures in which the washes are identical to those above except for the temperature of the final two 30 min washes in 0.2X SSC, 0.5% SDS was increased to 60°C. Another preferred set of highly stringent conditions uses two final washes in 0.1X SSC, 0.1% SDS at 65°C. An additional set of stringent conditions include hybridization at 0.1X SSC, 0.1% SDS, 65°C and washed with 2X SSC, 0.1% SDS followed by 0.1X SSC, 0.1% SDS, for example.

Hybridization requires that the two nucleic acids contain complementary sequences, although depending on the stringency of the hybridization, mismatches between bases are possible. The appropriate stringency for hybridizing nucleic acids depends on the length of the nucleic acids and the degree of complementation, variables well known in the art. The greater the degree of similarity or homology between two nucleotide sequences, the greater the value of T_m for hybrids of nucleic acids having those sequences. The relative stability (corresponding to higher T_m) of nucleic acid hybridizations decreases in the following order: RNA:RNA, DNA:RNA, DNA:DNA. For hybrids of greater than 100 nucleotides in length, equations for calculating T_m have been derived (see Sambrook et al., *supra*, 9.50-9.51). For hybridizations with shorter nucleic acids, i.e., oligonucleotides, the position of mismatches becomes more important, and the length of the oligonucleotide determines its specificity (see Sambrook et al., *supra*, 11.7-11.8). In one embodiment the length for a hybridizable nucleic acid is at least about 10 nucleotides. Preferably a minimum length for a hybridizable nucleic acid is at least about 15 nucleotides; more preferably at least about 20 nucleotides; and most preferably the length is at least about 30 nucleotides. Furthermore, the skilled artisan will recognize that the temperature and wash solution salt concentration may be adjusted as necessary according to factors such as length of the probe.

A "substantial portion" of an amino acid or nucleotide sequence is that portion comprising enough of the amino acid sequence of a 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 general, a sequence of ten or more contiguous amino acids or thirty or more nucleotides is necessary in order to identify putatively 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 used in sequence-dependent methods of gene identification (e.g., Southern hybridization) and isolation (e.g., *in situ* hybridization of bacterial 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 molecule comprising the primers. Accordingly, a "substantial portion" of a nucleotide sequence comprises enough of the sequence to specifically identify and/or isolate a nucleic acid molecule comprising the sequence.

The instant specification teaches partial or complete amino acid and nucleotide sequences encoding one or more particular microbial proteins and promoters. The skilled artisan, having the benefit of the sequences as reported herein, may now use all or a substantial portion of the disclosed sequences for purposes known to those skilled in this art. Accordingly, the instant invention comprises the complete sequences as reported in the accompanying Sequence Listing, as well as substantial portions of those sequences as defined above.

The term "oligonucleotide" refers to a nucleic acid, generally of at least 18 nucleotides, that is hybridizable to a genomic DNA molecule, a cDNA molecule, or an mRNA molecule. In one embodiment, a labeled oligonucleotide can be used as a "probe" to detect the presence of a nucleic acid according to the invention. Thus, the term "probe" refers to a single-stranded nucleic acid molecule that can base pair with a complementary single-stranded target nucleic acid to form a double-stranded molecule. The term "label" will refer to any conventional molecule which can be readily attached to mRNA or DNA and which can

produce a detectable signal, the intensity of which indicates the relative amount of hybridization of the labeled probe to the DNA fragment.

The term "complementary" is used to describe the relationship between nucleotide bases that are capable of hybridizing to one another.

5 For example, with respect to DNA, adenosine is complementary to thymine and cytosine is complementary to guanine. Accordingly, the instant invention also includes isolated nucleic acid molecules that are complementary to the complete sequences as reported in the accompanying Sequence Listing, as well as those substantially similar
10 nucleic acid sequences.

The term "percent identity", as known in the art, is a relationship between two or more polypeptide sequences or two or more polynucleotide sequences, as determined by comparing the sequences. In the art, "identity" also means the degree of sequence relatedness
15 between polypeptide or polynucleotide sequences, as the case may be, as determined by the match between strings of such sequences. "Identity" and "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
20 (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.)
25 Stockton: NY (1991). Preferred methods to determine identity are designed to give the best match between the sequences tested. Methods to determine identity and similarity are codified in publicly available computer programs. Sequence alignments and percent identity calculations may be performed using the Megalign program of the
30 LASERGENE bioinformatics computing suite (DNASTAR Inc., Madison, WI). Multiple alignment of the sequences is performed using the Clustal method of alignment (Higgins and Sharp, *CABIOS*. 5:151-153 (1989)) with default parameters (GAP PENALTY=10, GAP LENGTH PENALTY=10). Default parameters for pairwise alignments using the Clustal method are:
35 KTUPLE 1, GAP PENALTY=3, WINDOW=5 and DIAGONALS SAVED=5.

Suitable nucleic acid molecules (isolated polynucleotides of the present invention) 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 molecules encode amino acid sequences that are about 85% identical to the amino acid sequences reported herein. More preferred nucleic acid molecules encode amino acid sequences that are at least about 90% identical to the amino acid sequences reported herein. Most preferred are nucleic acid molecules that encode amino acid sequences that are at least about 95% identical to the amino acid sequences reported herein. Suitable nucleic acid molecules 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.

Likewise, suitable promoter regions (isolated polynucleotides of the present invention) encode promoter regions that are at least about 70% identical, preferably at least about 75% identical, and more preferably at least about 80% identical to the nucleotide sequences reported herein. Preferred nucleic acid molecules are about 85% identical to the nucleotide sequences reported herein, more preferred nucleic acid molecules are at least about 90% identical, and most preferred are nucleic acid molecules at least about 95% identical to the nucleotide sequences reported herein. Suitable promoter regions not only have the above homologies but typically are at least 50 nucleotides in length, more preferably at least 100 nucleotides in length, more preferably at least 250 nucleotides in length, and more preferably at least 500 nucleotides in length.

“Codon degeneracy” refers to the nature in the genetic code permitting variation of the nucleotide sequence without affecting the amino acid sequence of an encoded polypeptide. Accordingly, the instant invention relates to any nucleic acid molecule that encodes all or a substantial portion of the amino acid sequence encoding the instant microbial polypeptides as set forth in SEQ ID NOs:16 and 26. The skilled artisan is well aware of the “codon-bias” exhibited by a specific host cell in usage of nucleotide codons to specify a given amino acid. Therefore, when synthesizing a gene for improved expression in a host cell, it is desirable to design the gene such that its frequency of codon usage approaches the frequency of preferred codon usage of the host cell.

“Chemically synthesized”, as related to a sequence of DNA, means that the component nucleotides were assembled *in vitro*. Manual chemical synthesis of DNA may be accomplished using well-established procedures; or, automated chemical synthesis can be performed using one of a number of commercially available machines. “Synthetic genes” can be assembled from oligonucleotide building blocks that are chemically synthesized using procedures known to those skilled in the art. These building blocks are ligated and annealed to form gene segments that are then enzymatically assembled to construct the entire gene. Accordingly, the genes can be tailored for optimal gene expression based on optimization of nucleotide sequence to reflect the codon bias of the host cell. The skilled artisan appreciates the likelihood of successful gene expression if codon usage is biased towards those codons favored by the host. Determination of preferred codons can be based on a survey of genes derived from the host cell, where sequence information is available.

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

“Coding sequence” refers to a DNA sequence that codes for a specific amino acid sequence.

“Suitable regulatory sequences” refer to transcriptional and translational 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, translation leader sequences, introns, polyadenylation recognition sequences, RNA processing sites, effector binding sites and stem-loop structures.

“Promoter” refers to a DNA sequence capable of controlling the expression of a coding sequence or functional RNA. In general, a coding sequence is located 3' to a promoter sequence. Promoters may be derived in their entirety from a native gene, or be composed of different elements derived from different promoters found in nature, or even comprise synthetic DNA segments. It is understood by those skilled in the art that different promoters may direct the expression of a gene in different tissues or cell types, or at different stages of development, or in response to different environmental 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 term “mutant promoter” is defined herein as a promoter having a nucleotide sequence comprising a substitution, deletion, and/or insertion of one or more nucleotides relative to the parent promoter, wherein the mutant promoter has more or less promoter activity than the corresponding parent promoter. The term “mutant promoter” will encompass natural variants and *in vitro* generated variants obtained using methods well known in the art (e.g., classical mutagenesis, site-directed mutagenesis and “DNA shuffling”).

The term “3' non-coding sequences” or “transcription terminator” refers 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
5 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
10 RNA that is without introns and that 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
15 part of a target primary transcript or mRNA and that blocks the expression of a target gene (U.S. 5,107,065; 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
20 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 molecule so that the function of one is affected by the other. For example, a promoter is operably linked with a
25 coding sequence when it is capable of affecting the expression of that coding sequence (i.e., the coding sequence is under the transcriptional control of the promoter). Coding sequences can be operably linked to regulatory sequences in sense or antisense orientation.

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

“Introns” are sequences of non-coding DNA found in gene sequences (either in the coding region, 5' non-coding region, or 3' non-
35 coding region) in most eukaryotes. Their full function is not known; however, some enhancers are located in the introns (Giacopelli F. et al., *Gene Expr.* 11:95-104 (2003)). These intron sequences are transcribed,

but removed from within the pre-mRNA transcript before the mRNA is translated into a protein. This process of intron removal occurs by self-splicing of the sequences (exons) on either side of the intron.

The term "altered biological activity" will refer to an activity,
5 associated with a protein encoded by a nucleotide sequence which can be measured by an assay method, where that activity is either greater than or less than the activity associated with the native sequence. "Enhanced biological activity" refers to an altered activity that is greater than that associated with the native sequence. "Diminished biological activity" is an
10 altered activity that is less than that associated with the native sequence.

"Transformation" refers to the transfer of a nucleic acid molecule 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
15 organisms containing the transformed nucleic acid molecules are referred to as "transgenic" or "recombinant" or "transformed" organisms.

The terms "plasmid", "vector" and "cassette" refer to an extra chromosomal element often carrying genes that are not part of the central metabolism of the cell, and usually in the form of circular double-stranded
20 DNA fragments. Such elements may be autonomously replicating sequences, genome integrating sequences, phage or nucleotide sequences, linear or circular, of a single- or double-stranded DNA or RNA, derived from any source, in which a number of nucleotide sequences have been joined or recombined into a unique construction which is capable of
25 introducing a promoter fragment and DNA sequence for a selected gene product along with appropriate 3' untranslated sequence into a cell.

"Transformation cassette" refers to a specific vector containing a foreign gene and having elements in addition to the foreign gene that facilitates transformation of a particular host cell. "Expression cassette" refers to a
30 specific vector containing a foreign gene and having elements in addition to the foreign gene that allow for enhanced expression of that gene in a foreign host.

The term "sequence analysis software" refers to any computer algorithm or software program that is useful for the analysis of nucleotide
35 or amino acid sequences. "Sequence analysis software" may be commercially available or independently developed. Typical sequence analysis software will 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); and 4.) the FASTA program incorporating the Smith-Waterman algorithm (W. R. Pearson, *Comput. Methods Genome Res.*, [Proc. Int. Symp.] (1994), Meeting Date 1992, 111-20. Suhai, Sandor, Ed. Plenum: New York, NY). Within the context of this application it will be understood that where sequence analysis software is used for analysis, that the results of the analysis will be based on the "default values" of the program referenced, unless otherwise specified. As used herein "default values" will mean any set of values or parameters that originally load with the software when first initialized.

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 (1987).

Identification Of The *gpd* And *gpm* Genes In *Yarrowia lipolytica*

The present invention identifies the partial sequence of a glyceraldehyde-3-phosphate dehydrogenase (*gpd*) gene (wherein ~115 amino acids of the C-terminus of the encoded protein are not disclosed herein) and the complete sequence of the phosphoglycerate mutase (*gpm*) gene contained within the *Yarrowia lipolytica* genome.

Comparison of the partial *gpd* nucleotide base and deduced amino acid sequences (SEQ ID NOs:25 and 26) to public databases reveals that the most similar known sequences are about 81% identical to the amino acid sequence of *gpd* reported herein over a length of 215 amino acids using a Smith-Waterman alignment algorithm (W. R. Pearson, *supra*). Preferred amino acid fragments are at least about 70%-80% identical to the sequences herein, where those sequences that are 85%-90% identical are particularly suitable and those sequences that are about 95% identical are most preferred. Similarly, preferred *gpd* encoding nucleic acid sequences corresponding to the instant ORF are those encoding active

proteins and which are at least about 70%-80% identical to the nucleic acid sequences of *gpd* reported herein, where those sequences that are 85%-90% identical are particularly suitable and those sequences that are about 95% identical are most preferred.

5 Comparison of the *gpm* nucleotide base and deduced amino acid sequences (SEQ ID NOs:15 and 16) to public databases reveals that the most similar known sequences are about 71% identical to the amino acid sequence of *gpm* reported herein over a length of 216 amino acids using a Smith-Waterman alignment algorithm (W. R. Pearson, *supra*). Preferred
10 amino acid fragments are at least about 70%-80% identical to the sequences herein, where those sequences that are 85%-90% identical are particularly suitable and those sequences that are about 95% identical are most preferred. Similarly, preferred *gpm* encoding nucleic acid sequences corresponding to the instant ORF are those encoding active proteins and
15 which are at least about 70%-80% identical to the nucleic acid sequences of *gpm* reported herein, where those sequences that are 85%-90% identical are particularly suitable and those sequences that are about 95% identical are most preferred.

Identification Of Natural Promoter Regions In *Yarrowia lipolytica*

20 The present invention also identifies putative promoter regions that naturally regulate GPD and GPM in *Yarrowia lipolytica*. These putative promoter regions have been identified as useful for driving expression of any suitable coding region of interest in a transformed yeast cell.

25 In the context of the present invention, a promoter useful in an oleaginous yeast should meet the following criteria:

- 1.) Strength. A strong yeast promoter is a necessary premise for a high expression level, and the low copy number of the ars18 (Fournier, P. et al. *Yeast* 7:25-36 (1991)) based expression vectors or chimeric genes integrated into the genome makes
30 this demand even more important when *Y. lipolytica* is used as the host organism.
- 2.) Activity in a medium suitable for expression of the coding region of interest, and high enzymatic activity of that coding region of interest.
- 35 3.) pH Tolerance. If the coding region of interest is known to be produced only in e.g., an acidic environment, then the promoter operably linked to said coding region of interest must

function at the appropriate pH. pH tolerance is of course limited by the tolerance of the host organism.

- 4.) Inducibility. A tightly regulated yeast promoter makes it possible to separate the growth stage from the expression stage, thereby enabling expression of products that are known to inhibit cell growth.
- 5.) Activity in the stationary phase of growth in oleaginous yeast hosts for accumulation of PUFAs.

Additionally, it is preferable for novel yeast promoters to possess differences in activity with respect to the known *Yarrowia lipolytica* TEF and/or XPR2 promoters (U.S. 4,937,189; EP220864; EP832258; U.S. 6,265,185). A comparative study of the TEF promoter and the GPD and GPM promoters of the instant invention is provided in Example 7. It is shown that the yeast promoters of the invention have improved activity compared to the TEF promoter. The promoter region of the instant GPD gene is contained within several nucleic acid molecules, specifically, SEQ ID NOs: 23, 24 and 43. In one embodiment, the GPD promoter will comprise nucleotides -500 to +1 of SEQ ID NO:43 (wherein the 'A' position of the 'ATG' translation initiation codon is designated as +1), thereby permitting relatively strong promoter activity; in alternate embodiments, the -100 to +1 region of SEQ ID NO:43 should be sufficient for basal activity of the promoter.

The GPM promoter region of the instant invention is contained in several nucleic acid molecules disclosed herein, including SEQ ID NOs:27, 28 and 44. In one embodiment, the GPM promoter will comprise nucleotides -500 to +1 of SEQ ID NO:44 (wherein the 'A' position of the 'ATG' translation initiation codon is designated as +1), thereby permitting relatively strong promoter activity; alternatively, the -100 to +1 region of SEQ ID NO:44 should be sufficient for basal activity of the promoter.

The promoter regions of the invention may comprise additional nucleotides to those specified above. For example, the promoter sequences of the invention may be constructed on the basis of the DNA sequence presented as SEQ ID NO:23 or SEQ ID NO:27 (SEQ ID NOs:43 and 44 are subsequences thereof, respectively). It should be recognized that promoter fragments of various diminishing lengths may have identical promoter activity, since the exact boundaries of the regulatory sequences have not been completely defined.

In alternate embodiments mutant promoters may be constructed, wherein the DNA sequence of the promoter has one or more nucleotide substitutions (i.e., deletions, insertions, substitutions, or addition of one or more nucleotides in the sequence) which do not effect (in particular impair) the yeast promoter activity. Regions that can be modified without significantly affecting the yeast promoter activity can be identified by deletion studies. A mutant promoter of the present invention has at least about 20%, preferably at least about 40%, more preferably at least about 60%, more preferably at least about 80%, more preferably at least about 90%, more preferably at least about 100%, more preferably at least about 200%, more preferably at least about 300% and most preferably at least about 400% of the promoter activity of the GPD or GPM promoter regions described herein as SEQ ID NOs:43 and 44.

Methods for mutagenesis are well known in the art and suitable for the generation of mutant promoters. For example, *in vitro* mutagenesis and selection, PCR based random mutagenesis, site-directed mutagenesis, or other means can be employed to obtain mutations of the naturally occurring promoters and genes of the instant invention. This would permit production of a putative promoter having a more desirable level of promoter activity in the host cell, or production of a polypeptide having more desirable physical and kinetic parameters for function in the host cell.

If desired, the regions of a nucleotide of interest important for promoter or enzymatic activity, respectively, can be determined through routine mutagenesis, expression of the resulting mutant promoters or polypeptides and determination of their activities. Mutants may include deletions, insertions and point mutations, or combinations thereof. A typical functional analysis begins with deletion mutagenesis to determine either: 1.) the minimum portion of the putative promoter necessary for activity; or 2.) the N- and C-terminal limits of the protein necessary for function. Subsequently, internal deletions, insertions or point mutants are made to further determine regions necessary for function. Other techniques such as cassette mutagenesis or total synthesis also can be used.

Deletion mutagenesis of a coding sequence is accomplished, for example, by using exonucleases to sequentially remove the 5' or 3' coding regions. Kits are available for such techniques. After deletion, the coding

region is completed by ligating oligonucleotides containing start or stop codons to the deleted coding region after 5' or 3' deletion, respectively. Alternatively, oligonucleotides encoding start or stop codons are inserted into the coding region by a variety of methods including site-directed mutagenesis, mutagenic PCR or by ligation onto DNA digested at existing restriction sites.

Internal deletions in a putative promoter region or within a coding sequence can similarly be made through a variety of methods including the use of existing restriction sites in the DNA, by use of mutagenic primers via site-directed mutagenesis or mutagenic PCR. Insertions are made through methods such as linker-scanning mutagenesis, site-directed mutagenesis or mutagenic PCR, while point mutations are made through techniques such as site-directed mutagenesis or mutagenic PCR.

Chemical mutagenesis also can be used for identifying regions of a putative promoter region or polypeptide important for activity. A mutated construct is expressed, and the ability of the resulting altered promoter or protein, respectively, is assayed. Such structure-function analysis can determine which regions may be deleted, which regions tolerate insertions, and which point mutations allow the mutant promoter or protein to function in substantially the same way as the native promoter or protein. All such mutant promoters and nucleotide sequences encoding polypeptides that are derived from the instant promoters and genes described herein are within the scope of the present invention. Isolation Of Homologs To The *gpd* And *gpm* Genes And Putative Promoter Regions

It will be appreciated by a person of skill in the art that the promoter regions and genes of the present invention have homologs in a variety of yeast species; and, the use of the promoters and genes for heterologous gene expression are not limited to those promoters and genes derived from *Yarrowia lipolytica*, but extend to homologs in other yeast species. For example, the invention encompasses homologs derived from oleaginous genera including, but not limited to: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*; examples of preferred species within these genera include: *Rhodospiridium toruloides*, *Lipomyces starkeyii*, *L. lipoferus*, *Candida revkaufi*, *C. pulcherrima*, *C. tropicalis*, *C. utilis*, *Trichosporon pullans*, *T. cutaneum*, *Rhodotorula glutinus* and *R. graminis*.

Homology typically is measured using sequence analysis software, wherein the term "sequence analysis software" refers to any computer algorithm or software program (commercially available or independently developed) that is useful for the analysis of nucleotide or amino acid sequences. In general, such computer software matches similar sequences by assigning degrees of homology to various substitutions, deletions and other modifications.

As is well known in the art, isolation of homologous promoter regions or genes using sequence-dependent protocols is readily possible using various techniques. Examples of sequence-dependent protocols include, but are not limited to: 1.) methods of nucleic acid hybridization; 2.) methods of DNA and RNA amplification, as exemplified by various uses of nucleic acid amplification technologies [e.g., polymerase chain reaction (PCR), Mullis et al., U.S. Patent 4,683,202; ligase chain reaction (LCR), Tabor, S. et al., *Proc. Acad. Sci. USA* 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, putative promoter regions or genes encoding similar proteins or polypeptides to those of the instant invention could be isolated directly by using all or a portion of the instant nucleic acid molecules as DNA hybridization probes to screen libraries from any desired microbe using methodology well known to those skilled in the art. Specific oligonucleotide probes based upon the instant nucleic acid sequences can be designed and synthesized by methods known in the art (Maniatis, *supra*). Moreover, the entire sequences can be used directly to synthesize DNA probes by methods known to the skilled artisan (e.g., random primers DNA labeling, nick translation, or end-labeling techniques), or RNA probes using available *in vitro* transcription systems. In addition, specific primers can be designed and used to amplify a part of (or full-length of) the instant sequences. The resulting amplification products can be labeled directly during amplification reactions or labeled after amplification reactions, and used as probes to isolate full-length DNA fragments under conditions of appropriate stringency.

Typically, in PCR-type amplification techniques, the primers have different sequences and are not complementary to each other. Depending on the desired test conditions, the sequences of the primers should be

designed to provide for both efficient and faithful replication of the target nucleic acid. Methods of PCR primer design are common and well known in the art (Thein and Wallace, "The use of 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; and 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 the instant sequences may be used in polymerase chain reaction protocols to amplify longer nucleic acid molecules encoding homologous polynucleotides from DNA or RNA. The polymerase chain reaction may also be performed on a library of cloned nucleic acid molecules wherein the sequence of one primer is derived from the instant nucleic acid molecules, and the sequence of the other primer takes advantage of the presence of the polyadenylic acid tracts to the 3' end of the mRNA precursor encoding microbial genes.

Alternatively, the instant sequences may be employed as 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 nucleotide sequence of interest, and a specific hybridization method. Probes of the present invention are typically single-stranded nucleic acid sequences that are complementary to the nucleic acid sequences to be detected. Probes are "hybridizable" to the nucleic acid sequence to be detected. The probe length can vary from 5 bases to tens of thousands of bases, and will depend upon the specific test to be done. Typically a probe length of about 15 bases to about 30 bases is suitable. Only part of the probe molecule need be complementary to the nucleic acid sequence to be detected. In addition, the complementarity between the probe and the target sequence need not be perfect. Hybridization does occur between imperfectly complementary molecules with the result that a certain fraction of the bases in the hybridized region are not paired with the proper complementary base.

Hybridization methods are well defined. Typically the probe and sample must be mixed under conditions that will permit nucleic acid hybridization. This involves contacting the probe and sample in the presence of an inorganic or organic salt under the proper concentration and temperature conditions. The probe and sample nucleic acids must be

in contact for a long enough time that any possible hybridization between the probe and sample nucleic acid may occur. The concentration of probe or target in the mixture will determine the time necessary for hybridization to occur. The higher the probe or target concentration, the shorter the hybridization incubation time needed. Optionally, a chaotropic agent may be added. The chaotropic agent stabilizes nucleic acids by inhibiting nuclease activity. Furthermore, the chaotropic agent allows sensitive and stringent hybridization of short oligonucleotide probes at room temperature (Van Ness and Chen, *Nucl. Acids Res.* 19:5143-5151 (1991)). Suitable chaotropic agents include guanidinium chloride, guanidinium thiocyanate, sodium thiocyanate, lithium tetrachloroacetate, sodium perchlorate, rubidium tetrachloroacetate, potassium iodide and cesium trifluoroacetate, among others. Typically, the chaotropic agent will be present at a final concentration of about 3 M. If desired, one can add formamide to the hybridization mixture, typically 30-50% (v/v).

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

Recombinant Expression In Yeast

Initiation control regions or promoter regions that are useful to drive expression of a coding gene of interest in the desired host cell are selected from those derived from the upstream portion of the *gpd* and *gpm* genes (SEQ ID NOs:25 and 15, respectively). The promoter regions may be identified from the upstream sequences of *gpd* and *gpm* genes and

their homologs and isolated according to common methods (Maniatis, *supra*). Once the promoter regions are identified and isolated, they may be operably linked to a coding region of interest to be expressed in a suitable expression vector. These chimeric genes may then be expressed
5 in natural host cells and heterologous host cells, particularly in the cells of oleaginous yeast hosts. Thus, one aspect of the present invention provides a recombinant expression vector comprising a yeast promoter of the invention.

In a further aspect, the invention provides a method of expressing a
10 coding region of interest in a transformed yeast cell, wherein a transformed cell is provided having a chimeric gene comprising: (i) a GPD or GPM promoter region and (ii) a coding region of interest expressible in the host, wherein the promoter region is operably linked to the coding region of interest; and the transformed cell is grown under conditions
15 wherein the chimeric gene is expressed. The polypeptide so produced can optionally be recovered from the culture.

Microbial expression systems and expression vectors are well known to those skilled in the art. Any of these could be used to construct chimeric genes comprising the promoter regions derived from the *gpm* and
20 *gpd* genes for production of any specific coding region of interest suitable for expression in a desirable yeast host cell. These chimeric genes could then be introduced into appropriate microorganisms by integration via transformation to provide high-level expression of the enzymes upon induction. Alternatively, the promoters can be cloned into a plasmid that is
25 capable of transforming and replicating itself in the preferred yeast host cell. The coding region of interest to be expressed can then be cloned downstream from the promoter. Once the recombinant host is established, gene expression can be accomplished by growing the cells under suitable conditions (*infra*).

30 Suitable Coding Regions Of Interest

Useful chimeric genes will include the promoter region of either of the *gpd* and *gpm* genes as defined herein or a mutant promoter thereof, operably linked to a suitable coding region of interest to be expressed in a preferred host cell.

35 Coding regions of interest to be expressed in the recombinant yeast host may be either endogenous to the host or heterologous and must be compatible with the host organism. Genes encoding proteins of

commercial value are particularly suitable for expression. For example, suitable coding regions of interest may include (but are not limited to) those encoding viral, bacterial, fungal, plant, insect, or vertebrate coding regions of interest, including mammalian polypeptides. Further, these coding regions of interest may be, for example, structural proteins, enzymes (e.g., oxidoreductases, transferases, hydrolyases, lyases, isomerases, ligases), or peptides. A non-limiting list includes genes encoding enzymes such as aminopeptidases, amylases, carbohydrases, carboxypeptidases, catalyases, cellulases, chitinases, cutinases, cyclodextrin glycosyltransferases, deoxyribonucleases, esterases, α -galactosidases, β -glucanases, β -galactosidases, glucoamylases, α -glucosidases, β -glucosidases, invertases, laccases, lipases, mannosidases, mutanases, oxidases, pectinolytic enzymes, peroxidases, phospholipases, phytases, polyphenoloxidases, proteolytic enzymes, ribonucleases, transglutaminases or xylanases.

Preferred in the present invention in some embodiments are coding regions of the enzymes involved in the production of microbial oils, including ω -6 and ω -3 fatty acids. Many microorganisms, including algae, bacteria, molds and yeast, can synthesize PUFAs and omega fatty acids in the ordinary course of cellular metabolism. Particularly well-studied are fungi including *Schizochytrium aggregatum*, species of the genus *Thraustochytrium* and *Mortierella alpina*. Additionally, many dinoflagellates (Dinophyceae) naturally produce high concentrations of PUFAs. As such, a variety of genes involved in oil production have been identified through genetic means and the DNA sequences of some of these genes are publicly available (e.g., see GenBank Accession No.'s AY131238, Y055118, AY055117, AF296076, AF007561, L11421, NM_031344, AF465283, AF465282, AF465281, AF110510, AF419296, AB052086, AJ250735, AF126799, AF126798, AF199596, AF226273, AF320509, AB072976, AF489588, AJ510244, AF419297, AF07879, AF067654, AB022097, AF489589.1, AY332747, AAG36933, AF110509, AB020033, AAL13300, AF417244, AF161219, X86736, AF240777, AB007640, AB075526, AP002063, NP_441622, BAA18302, BAA02924, AAL36934, AF338466, AF438199, E11368, E11367, D83185, U90417, AF085500, AY504633, NM_069854, AF230693, AX464731, NM_119617, NM_134255, NM_134383, NM_134382, NM_068396, NM_068392, NM_070713, NM_068746 and NM_064685). Additionally, the patent

literature provides many additional DNA sequences of genes (and/or details concerning several of the genes above and their methods of isolation) involved in oil production. See, for example: U.S. 5,968,809 ($\Delta 6$ desaturases); U.S. 5,972,664 and U.S. 6,075,183 ($\Delta 5$ desaturases); WO 91/13972 and U.S. 5,057,419 ($\Delta 9$ desaturases); WO 93/11245 ($\Delta 15$ desaturases); WO 94/11516, U.S. 5,443,974 and WO 03/099216 ($\Delta 12$ desaturases); U.S. 2003/0196217 A1 ($\Delta 17$ desaturase); WO 00/12720 and U.S. 2002/0139974A1 (elongases), each of which is herein incorporated by reference in its entirety.

10 Components Of Vectors/ DNA Cassettes

Vectors or DNA cassettes useful for the transformation of suitable host cells are well known in the art. The specific choice of sequences present in the construct is dependent upon the desired expression products (*supra*), the nature of the host cell, and the proposed means of separating transformed cells versus non-transformed cells. Typically, however, the vector 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 5' of the gene that controls transcriptional initiation and a region 3' of the DNA fragment that controls transcriptional termination. It is most preferred when both control regions are derived from genes from the transformed host cell, although it is to be understood that such control regions need not be derived from the genes native to the specific species chosen as a production host.

25 Nucleotide sequences surrounding the translational initiation codon 'ATG' have been found to affect expression in yeast cells. If the desired polypeptide is poorly expressed in yeast, the nucleotide sequences of exogenous genes can be modified to include an efficient yeast translation initiation sequence motif to obtain optimal gene expression. For expression in yeast, this can be done by site-directed mutagenesis of an inefficiently expressed gene to include the favored translation initiation motif.

35 The termination region can be derived from the 3' region of the gene from which the initiation region was obtained or from a different gene. A large number of termination regions are known and function satisfactorily in a variety of hosts (when utilized both in the same and different genera and species from where they were derived). The

termination region usually is selected more as a matter of convenience rather than because of any particular property. Preferably, the termination region is derived from a yeast gene, particularly *Saccharomyces*, *Schizosaccharomyces*, *Candida*, *Yarrowia* or *Kluyveromyces*. The 3'-
5 regions of mammalian genes encoding γ -interferon and α -2 interferon are also known to function in yeast. Termination control regions may also be derived from various genes native to the preferred hosts. Optionally, a termination site may be unnecessary; however, it is most preferred if included.

10 As one of skill in the art is aware, merely inserting a chimeric gene into a cloning vector does not ensure that it will be successfully expressed at the level needed. In response to needs for high expression rates, many specialized expression vectors have been created by manipulating a number of different genetic elements that control aspects of transcription,
15 translation, protein stability, oxygen limitation and secretion from the host cell. More specifically, some of the molecular features that have been manipulated to control gene expression include: 1.) the nature of the relevant transcriptional promoter and terminator sequences; 2.) the number of copies of the cloned gene and whether the gene is plasmid-
20 borne or integrated into the genome of the host cell; 3.) the final cellular location of the synthesized foreign protein; 4.) the efficiency of translation in the host organism; 5.) the intrinsic stability of the cloned gene protein within the host cell; and 6.) the codon usage within the cloned gene, such that its frequency approaches the frequency of preferred codon usage of
25 the host cell. Each of these types of modifications are encompassed in the present invention, as means to further optimize expression of a chimeric gene comprising a promoter region of either of the *gpd* and *gpm* genes as defined herein or a mutant promoter thereof, operably linked to a suitable coding region of interest.

30 Transformation Of Yeast Cells

Once an appropriate chimeric gene has been constructed that is suitable for expression in a yeast cell, it is placed in a plasmid vector capable of autonomous replication in a host cell or it is directly integrated into the genome of the host cell. Integration of expression cassettes can
35 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.

Where two or more genes are expressed from separate replicating vectors, it is desirable that each vector has a different means of selection and should lack homology to the other constructs 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 can be experimentally determined so that all introduced genes are expressed at the necessary levels to provide for synthesis of the desired products.

Constructs comprising a coding region 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, microinjection, or any other method that introduces the gene of interest into the host cell. More specific teachings applicable for oleaginous yeast (i.e., *Yarrowia lipolytica*) include U.S. Patent No's. 4,880,741 and 5,071,764 and Chen, D. C. et al. (*Appl Microbiol Biotechnol.* 48(2):232-235-(1997)).

For convenience, a host cell that has been manipulated by any method to take up a DNA sequence (e.g., an expression cassette) will be referred to as "transformed" or "recombinant" herein. The transformed host 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 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 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: 1.) 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
5 luciferin to a light-emitting product); or 2.) 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
10 selected, for example, visually, or by techniques such as FACS or panning using antibodies. For selection of yeast transformants, any marker that functions in yeast may be used. Preferred for use herein are resistance to kanamycin, hygromycin and the aminoglycoside G418, as well as ability to grow on media lacking uracil or leucine.

15 Techniques To Up-Regulate Expression Of A Chimeric Gene
Comprising A GPD Or GPM Promoter Operably Linked To A Coding
Region Of Interest

Additional copies a particular coding region of interest (operably linked to a promoter of the instant invention) may be introduced into the
20 host to increase expression. Expression of the coding region of interest also can be increased by removing/deleting destabilizing sequences from either the mRNA or the encoded protein, or by adding stabilizing sequences to the mRNA (U.S. 4,910,141).

Yet another approach to increase expression of a coding region of
25 interest is to increase the translational efficiency of the encoded mRNAs by replacement of codons in the native gene with those for optimal gene expression in the selected host microorganism. As will be appreciated by one skilled in the art, use of host preferred codons can substantially enhance the expression of the foreign gene encoding the polypeptide. In
30 general, host preferred codons can be determined within a particular host species of interest by examining codon usage in proteins (preferably those expressed in the largest amount) and determining which codons are used with highest frequency. Then, the coding sequence for a polypeptide of
35 interest can be synthesized in whole or in part using the codons preferred in the host species.

Preferred Hosts

Preferred host cells for expression of the instant genes and coding regions of interest operably linked to the instant promoter molecules herein are yeast cells (where oleaginous yeast are most preferred where the
 5 desired use is for the production of microbial oils, *infra*). Oleaginous yeast are naturally capable of oil synthesis and accumulation, wherein the oil can comprise greater than about 25% of the cellular dry weight, more preferably greater than about 30% of the cellular dry weight, and most preferably greater than about 40% of the cellular dry weight. Genera
 10 typically identified as oleaginous yeast include, but are not limited to: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*. More specifically, illustrative oil-synthesizing yeast 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 #20362, ATCC #8862, ATCC #18944, ATCC #76982 and/or
 20 LGAM S(7)1 (Papanikolaou S., and Aggelis G., *Bioresour. Technol.* 82(1):43-9 (2002)). The *Y. lipolytica* strain designated as ATCC #76982 was the particular strain from which the GPD and GPM promoters and genes were isolated herein.

Industrial Production Using Transformed Yeast Expressing A SuitableCoding Region Of Interest

In general, media conditions which may be optimized for high-level expression of a particular coding region of interest include the type and amount of carbon source, the type and amount of nitrogen source, the carbon-to-nitrogen ratio, the oxygen level, growth temperature, pH, length
 30 of the biomass production phase and the time of cell harvest. Microorganisms of interest, such as oleaginous yeast, are grown in complex media (e.g., yeast extract-peptone-dextrose broth (YPD)) or a defined minimal media that lacks a component necessary for growth and thereby forces selection of the desired expression cassettes (e.g., Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI)).

Fermentation media in the present invention must contain a suitable carbon source. Suitable carbon sources may include, but are not limited

to: monosaccharides (e.g., glucose, fructose), disaccharides (e.g., lactose, sucrose), oligosaccharides, polysaccharides (e.g., starch, cellulose or mixtures thereof), sugar alcohols (e.g., glycerol) or mixtures from renewable feedstocks (e.g., cheese whey permeate, cornsteep liquor, sugar beet molasses, barley malt). Additionally, carbon sources may include alkanes, fatty acids, esters of fatty acids, monoglycerides, diglycerides, triglycerides, phospholipids and various commercial sources of fatty acids including vegetable oils (e.g., soybean oil) and animal fats. Additionally, the carbon source may include one-carbon sources (e.g., carbon dioxide, methanol, formaldehyde, formate, carbon-containing amines) for which metabolic conversion into key biochemical intermediates has been demonstrated. Hence it is contemplated that the source of carbon utilized in the present invention may encompass a wide variety of carbon-containing sources and will only be limited by the choice of the host organism. Although all of the above mentioned carbon sources and mixtures thereof are expected to be suitable in the present invention, preferred carbon sources are sugars and/or fatty acids. Most preferred is 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 source (e.g., urea or glutamate). In addition to appropriate carbon and nitrogen sources, the fermentation media must also contain suitable minerals, salts, cofactors, buffers, vitamins, and other components known to those skilled in the art suitable for the growth of the microorganism.

Preferred growth media in the present invention are common commercially prepared media, such as Yeast Nitrogen Base (DIFCO Laboratories, Detroit, MI). Other defined or synthetic growth media may also be used and the appropriate medium for growth of the particular microorganism will be known by one skilled in the art of microbiology or fermentation science. A suitable pH range for the fermentation is typically between about pH 4.0 to pH 8.0, wherein pH 5.5 to pH 7.0 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.

Host cells comprising a suitable coding region of interest operably linked to the promoters of the present invention may be cultured using methods known in the art. For example, the cell may be cultivated by shake flask cultivation, small-scale or large-scale fermentation in

laboratory or industrial fermentors performed in a suitable medium and under conditions allowing expression of the coding region of interest.

Where commercial production of a product that relies on the instant genetic chimera is desired, a variety of culture methodologies may be applied. For example, large-scale production of a specific gene product over-expressed from a recombinant host may be produced by a batch, fed-batch or continuous fermentation process.

A batch fermentation process is a closed system wherein the media composition is fixed at the beginning of the process and not subject to further additions beyond those required for maintenance of pH and oxygen level during the process. Thus, at the beginning of the culturing process the media is inoculated with the desired organism and growth or metabolic activity is permitted to occur without adding additional sources (i.e., carbon and nitrogen sources) to the medium. In batch processes the metabolite and biomass compositions of the system change constantly up to the time the culture is terminated. In a typical batch process, cells proceed through a static lag phase to a high growth log phase and finally to a stationary phase, wherein the growth rate is diminished or halted. Left untreated, cells in the stationary phase will eventually die. A variation of the standard batch process is the fed-batch process, wherein the source is continually added to the fermentor over the course of the fermentation process. A fed-batch process is also suitable in the present invention. Fed-batch processes are useful when catabolite repression is apt to inhibit the metabolism of the cells or where it is desirable to have limited amounts of source in the media at any one time. Measurement of the source concentration in fed-batch systems is difficult and therefore may be estimated on the basis of the changes of measurable factors such as pH, dissolved oxygen and the partial pressure of waste gases (e.g., CO₂). Batch and fed-batch culturing methods are common and well known in the art and examples may be found in Thomas D. Brock in Biotechnology: A Textbook of Industrial Microbiology, 2nd ed., (1989) Sinauer Associates: Sunderland, MA; or Deshpande, Mukund V., *Appl. Biochem. Biotechnol.*, 36:227 (1992), herein incorporated by reference.

Commercial production may also be accomplished by a continuous fermentation process, wherein a defined media is continuously added to a bioreactor while an equal amount of culture volume is removed simultaneously for product recovery. Continuous cultures generally

maintain the cells in the log phase of growth at a constant cell density. Continuous or semi-continuous culture methods permit the modulation of one factor or any number of factors that affect cell growth or end product concentration. For example, one approach may limit the carbon source
5 and allow all other parameters to moderate metabolism. In other systems, a number of factors affecting growth may be altered continuously while the cell concentration, measured by media turbidity, is kept constant. Continuous systems strive to maintain steady state growth and thus the cell growth rate must be balanced against cell loss due to media being
10 drawn off the culture. Methods of modulating nutrients and growth factors for continuous culture processes, as well as techniques for maximizing the rate of product formation, are well known in the art of industrial microbiology and a variety of methods are detailed by Brock, *supra*.

DESCRIPTION OF PREFERRED EMBODIMENTS

15 Although the promoters of the present invention will be suitable for expression of any suitable coding region of interest in an oleaginous yeast, in a preferred embodiment the promoters will be utilized in the development of an oleaginous yeast that accumulates oils enriched in PUFAs. Toward this end, it is necessary to introduce and express e.g.,
20 desaturases and elongases that allow for the synthesis and accumulation of ω -3 and/or ω -6 fatty acids.

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
25 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 number of carbon (C) atoms and Y is the number of double bonds.

Generally, fatty acids are classified as saturated or unsaturated. The term "saturated fatty acids" refers to those fatty acids that have no
30 "double bonds" between their carbon backbone. In contrast, "unsaturated fatty acids" are *cis*-isomers that have "double bonds" along their carbon backbones. "Monounsaturated fatty acids" have only one "double bond" along the carbon backbone (e.g., usually between the 9th and 10th carbon atom as for palmitoleic acid (16:1) and oleic acid (18:1)), while
35 "polyunsaturated fatty acids" (or "PUFAs") have at least two double bonds along the carbon backbone (e.g., between the 9th and 10th, and 12th and

13th carbon atoms for linoleic acid (18:2); and between the 9th and 10th, 12th and 13th, and 15th and 16th for α -linolenic acid (18:3)).

“PUFAs” can be classified into two major families (depending on the position (*n*) of the first double bond nearest the methyl end of the fatty acid carbon chain). Thus, the “ ω -6 fatty acids” (ω -6 or *n*-6) have the first unsaturated double bond six carbon atoms from the omega (methyl) end of the molecule and additionally have a total of two or more double bonds, with each subsequent unsaturation occurring 3 additional carbon atoms toward the carboxyl end of the molecule. In contrast, the “ ω -3 fatty acids” (ω -3 or *n*-3) have the first unsaturated double bond three carbon atoms away from the omega end of the molecule and additionally have a total of three or more double bonds, with each subsequent unsaturation occurring 3 additional carbon atoms toward the carboxyl end of the molecule.

For the purposes of this disclosure, the omega-reference system will be 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). This nomenclature is shown below in Table 1, in the column titled “Shorthand Notation”. The remainder of the Table summarizes the common names of ω -3 and ω -6 fatty acids, the abbreviations that will be used throughout the remainder of the specification, and each compounds’ chemical name.

Table 1
Nomenclature Of Polyunsaturated Fatty Acids

Common Name	Abbreviation	Chemical Name	Shorthand Notation
Linoleic	LA	<i>cis</i> -9,12-octadecadienoic	18:2 ω -6
γ -Linoleic	GLA	<i>cis</i> -6, 9, 12-octadecatrienoic	18:3 ω -6
Dihomo- γ -Linoleic	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-	18:4 ω -3

		octadecatetraenoic	
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
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

Microbial Biosynthesis Of Fatty Acids

In general, lipid accumulation in oleaginous microorganisms is triggered in response to the overall carbon to nitrogen ratio present in the growth medium. When cells have exhausted available nitrogen supplies (e.g., when the carbon to nitrogen ratio is greater than about 40), the depletion of cellular adenosine monophosphate (AMP) leads to the cessation of AMP-dependent isocitrate dehydrogenase activity in the mitochondria and the accumulation of citrate, transport of citrate into the cytosol, and subsequent cleavage of the citrate by ATP-citrate lyase to yield acetyl-CoA and oxaloacetate. Acetyl-CoA is the principle building block for *de novo* biosynthesis of fatty acids. The first committed step of fatty acid biosynthesis is the synthesis of malonyl-CoA, produced via carboxylation of acetyl-CoA. Fatty acid synthesis is catalyzed by a multi-enzyme fatty acid synthase complex and occurs by the condensation of eight two-carbon fragments (acetyl groups from acetyl-CoA) to form a 16-carbon saturated fatty acid, palmitate.

Palmitate is the precursor of longer chain saturated and unsaturated fatty acids (e.g., stearic (18:0), palmitoleic (16:1) and oleic (18:1) acids) through the action of elongases and desaturases present in the endoplasmic reticulum membrane. Palmitate and stearate are converted to their unsaturated derivatives, palmitoleic (16:1) and oleic (18:1) acids, respectively, by the action of a Δ^9 desaturase.

Biosynthesis Of Omega-3 And Omega-6 Fatty Acids

Simplistically, the metabolic process that converts LA to GLA, DGLA and ARA (the ω -6 pathway) and ALA to STA, ETA, EPA and DHA (the ω -3 pathway) involves elongation of the carbon chain through the addition of carbon atoms and desaturation of the molecule through the

addition of double bonds. This requires a series of special desaturation and elongation enzymes present in the endoplasmic reticulum membrane, hereinafter referred to as "PUFA biosynthetic pathway enzymes".

More specifically, "PUFA biosynthetic pathway enzymes" will refer to any of the following enzymes (and genes which encode said enzymes) associated with the 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 and/or an elongase. For further clarity within the present disclosure, the term "desaturase" refers to a polypeptide component of a multi-enzyme complex that can desaturate one or more fatty acids to produce a mono- or polyunsaturated fatty acid or precursor of interest. Thus, despite use of the omega-reference system 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 source using the delta-system. For example, a $\Delta 17$ desaturase will desaturate a fatty acid between the 17th and 18th carbon atom numbered from the carboxyl-terminal end of the molecule and can, for example, catalyze the conversion of ARA to EPA and/or DGLA to ETA. In contrast, the term "elongase" refers to a polypeptide component of a multi-enzyme complex that can elongate a fatty acid carbon chain to produce a mono- or polyunsaturated fatty acid that is 2 carbons longer than the fatty acid source that the elongase acts upon. This process of elongation occurs in a multi-step mechanism in association with fatty acid synthase, whereby CoA is the acyl carrier (Lassner et al., *The Plant Cell* 8:281-292 (1996)). Briefly, malonyl-CoA is condensed with a long-chain acyl-CoA to yield CO₂ and a β -ketoacyl-CoA (where the acyl moiety has been elongated by two carbon atoms). Subsequent reactions include reduction to β -hydroxyacyl-CoA, dehydration to an enoyl-CoA and a second reduction to yield the elongated acyl-CoA.

Synthesis of ω -6 fatty acids occurs in the following fashion: oleic acid (the first of the ω -6 fatty acids) is converted to LA (18:2) by the action of a $\Delta 12$ desaturase (Figure 10). Subsequent ω -6 fatty acids are produced as follows: 1.) LA is converted to GLA by the activity of a $\Delta 6$ desaturase; 2.) GLA is converted to DGLA by the action of an elongase; and 3.) DGLA is converted to ARA by the action of a $\Delta 5$ desaturase. In contrast, ω -3 fatty acids are all derived from linoleic acid (LA). Specifically: 1.) LA is converted to ALA by the action of a $\Delta 15$ desaturase; 2.) ALA is converted

to STA by the activity of a $\Delta 6$ desaturase; 3.) STA is converted to ETA by the activity of an elongase; and 4.) ETA is converted to EPA by the activity of a $\Delta 5$ desaturase. Alternatively, ETA and EPA can be produced from DGLA and ARA, respectively, by the activity of a $\Delta 17$ desaturase. EPA can be further converted to DHA by the activity of an elongase and a $\Delta 4$ desaturase.

Production Of PUFAs

As will be obvious to one skilled in the art, the particular functionalities required to be introduced into a host organism for production of a particular PUFA final product will depend on the host cell (and its native PUFA profile and/or desaturase profile), the availability of substrate and the desired end product(s). As shown in Figure 10, LA, GLA, DGLA, ARA, ALA, STA, ETA, EPA, DPA and DHA may all be produced in oleaginous yeast, by introducing various combinations of the following PUFA enzyme functionalities: 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, and/or an elongase. One skilled in the art will be able to identify various candidate genes encoding each of the above enzymes, according to publicly available literature (e.g., GenBank), the patent literature, and experimental analysis of microorganisms having the ability to produce PUFAs. Thus, a variety of desaturases and elongases are suitable as coding regions of interest in the present invention. These coding regions of interest could be operably linked to the the GPD and/or GPM promoters of the present invention or mutant promoters thereof, and used as chimeric genes for expression of various ω -6 and ω -3 fatty acids, using techniques well known to those skilled in the art (see, for example co-pending U.S. Patent Application No. 10/840579, herein incorporated entirely by reference). As such, the invention provides a method for the production of ω -3 and/or ω -6 fatty acids comprising:

- a) providing a transformed oleaginous yeast host cell comprising a chimeric gene, comprising:
 - 1) a promoter region of a gene selected from the group consisting of: the promoter region of a *gpm* gene and the promoter region of a *gpd* gene; and
 - 2) a coding region of interest expressible in the oleaginous yeast encoding an enzyme of a functional ω -3/ ω -6 fatty acid biosynthetic pathway;

wherein the promoter region and coding region are operably linked; and

- (b) contacting the host cell of step (a) under suitable growth conditions whereby one or more ω -3 or ω -6 fatty acids are produced.

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In preferred embodiments, the nucleic acid sequence of the promoter region is selected from the group consisting of: SEQ ID NOs:23, 27, 43 and 44, and subsequences and mutant promoters thereof; and the coding region of interest is any desaturase or elongase suitable for expression in the oleaginous yeast for the production of ω -3 or ω -6 fatty acids.

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For production of the greatest and the most economical yield of PUFAs, the transformed oleaginous yeast host cell is grown under conditions that optimize desaturase and elongase activities by optimizing expression of the chimeric genes of the present invention, wherein these chimeric genes comprise a promoter region of a *gpm* or *gpd* gene and a coding region of interest encoding a PUFA biosynthetic pathway enzyme.

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In the fermentation media, particular attention is given to several metal ions (e.g., Mn^{+2} , Co^{+2} , Zn^{+2} , Mg^{+2}) that promote synthesis of lipids and PUFAs (Nakahara, T. et al. *Ind. Appl. Single Cell Oils*, D. J. Kyle and R. Colin, eds. pp 61-97 (1992)).

20

The preferred "fermentable carbon source" for production of oleaginous yeast expressing various ω -6 and ω -3 fatty acids will include, but is not limited to: monosaccharides, oligosaccharides, polysaccharides, alkanes, fatty acids, esters of fatty acids, monoglycerides, diglycerides, triglycerides, carbon dioxide, methanol, formaldehyde, formate and carbon-containing amines.

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Typically, accumulation of high levels of PUFAs in oleaginous yeast cells requires a two-stage process, since the metabolic state must be "balanced" between growth and synthesis/storage of fats. Thus, most preferably, a two-stage fermentation process is necessary for the production of PUFAs in oleaginous yeast. In this approach, the first stage of the fermentation is dedicated to the generation and accumulation of cell mass and is characterized by rapid cell growth and cell division. In the second stage of the fermentation, it is preferable to establish conditions of nitrogen deprivation in the culture to promote high levels of lipid accumulation. The effect of this nitrogen deprivation is to reduce the

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effective concentration of AMP in the cells, thereby reducing the activity of the NAD-dependent isocitrate dehydrogenase of mitochondria. When this occurs, citric acid will accumulate, thus forming abundant pools of acetyl-CoA in the cytoplasm and priming fatty acid synthesis. Thus, this phase is characterized by the cessation of cell division followed by the synthesis of fatty acids and accumulation of oil. Although cells are typically grown at about 30 °C, some studies have shown increased synthesis of unsaturated fatty acids at lower temperatures (Yongmanitchai and Ward, *Appl. Environ. Microbiol.* 57:419-25 (1991)). Based on process economics, this temperature shift should likely occur after the first phase of the two-stage fermentation, when the bulk of the organisms' growth has occurred.

Purification Of PUFAs

The PUFAs produced in a host microorganism as described herein may be found as free fatty acids or in esterified forms such as acylglycerols, phospholipids, sulfolipids or glycolipids, and may be extracted from the host cell through a variety of means well-known in the art. One review of extraction techniques, quality analysis and acceptability standards for yeast lipids is that of Z. Jacobs (*Critical Reviews in Biotechnology* 12(5/6):463-491 (1992)). A brief review of downstream processing is also available by A. Singh and O. Ward (*Adv. Appl. Microbiol.* 45:271-312 (1997)).

In general, means for the purification of PUFAs may include extraction with organic solvents, sonication, supercritical fluid extraction (e.g., using carbon dioxide), saponification, and physical means such as presses, or combinations thereof. Of particular interest is extraction with methanol and chloroform in the presence of water (E. G. Bligh & W.J. Dyer, *Can. J. Biochem. Physiol.* 37:911-917 (1959)). Where desirable, the aqueous layer can be acidified to protonate negatively-charged moieties and thereby increase partitioning of desired products into the organic layer. After extraction, the organic solvents can be removed by evaporation under a stream of nitrogen. When isolated in conjugated forms, the products may be enzymatically or chemically cleaved to release the free fatty acid or a less complex conjugate of interest, and can then be subject to further manipulations to produce a desired end product. Desirably, conjugated forms of fatty acids are cleaved with potassium hydroxide.

If further purification is necessary, standard methods can be employed. Such methods may include extraction, treatment with urea, fractional crystallization, HPLC, fractional distillation, silica gel chromatography, high-speed centrifugation or distillation, or combinations of these techniques. Protection of reactive groups, such as the acid or alkenyl groups, may be done at any step through known techniques (e.g., alkylation or iodination). Methods used include methylation of the fatty acids to produce methyl esters. Similarly, protecting groups may be removed at any step. Desirably, purification of fractions containing GLA, STA, ARA, DHA and EPA may be accomplished by treatment with urea and/or fractional distillation.

EXAMPLES

The present invention is further defined in the following Examples. It should be understood that these Examples, while indicating preferred embodiments of the invention, are given by way of illustration only. From the above discussion and these Examples, one skilled in the art can ascertain the essential characteristics of this invention, and without departing from the spirit and scope thereof, can make various changes and modifications of the invention to adapt it to various usages and conditions.

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) (hereinafter "Maniatis"); 2.) T. J. Silhavy, M. L. Bennis, and L. W. Enquist, *Experiments with Gene Fusions*; Cold Spring Harbor Laboratory: Cold Spring Harbor, NY (1984); and 3.) Ausubel, F. M. et al., *Current Protocols in Molecular Biology*, published by Greene Publishing Assoc. and Wiley-Interscience (1987).

Materials and methods suitable for the maintenance and growth of microbial cultures are well known in the art. Techniques suitable for use in the following examples may be found as set out in Manual of Methods for General Bacteriology (Phillipp Gerhardt, R. G. E. Murray, Ralph N. Costilow, Eugene W. Nester, Willis A. Wood, Noel R. Krieg and G. Briggs Phillips, Eds), American Society for Microbiology: Washington, D.C. (1994)); or by Thomas D. Brock in Biotechnology: A Textbook of Industrial

Microbiology, 2nd ed., Sinauer Associates: Sunderland, MA (1989). All reagents, restriction enzymes and materials used for the growth and maintenance of microbial cells were obtained from Aldrich Chemicals (Milwaukee, WI), DIFCO Laboratories (Detroit, MI), GIBCO/BRL (Gaithersburg, MD), or Sigma Chemical Company (St. Louis, MO), unless otherwise specified.

A leucine autotrophic strain of *Yarrowia lipolytica* was purchased from the American Type Culture Collection (Rockville, MD; ATCC #76982) and used for functional assays. *Y. lipolytica* strains were usually grown at 28°C on YPD agar (1% yeast extract, 2% bactopectone, 2% glucose, 2% agar). For selection of transformants, minimal medium (0.17% yeast nitrogen base (DIFCO Laboratories) without ammonium sulfate or amino acids, 2% glucose, 0.1% proline, pH 6.1) was used. Supplements of adenine, leucine, lysine and/or uracil were added to a final concentration of 0.01%.

General molecular cloning was performed according to standard methods (Sambrook et al., *supra*). Oligonucleotides were synthesized by Sigma-Genosys (Spring, TX). Site-directed mutagenesis was performed using Stratagene's QuikChange™ Site-Directed Mutagenesis kit (San Diego, CA), per the manufacturer's instructions. When polymerase chain reaction (PCR) or site-directed mutagenesis was involved in subcloning, the constructs were sequenced to confirm that no errors had been introduced to the sequence. PCR products were cloned into Promega's pGEM-T-easy vector (Madison, WI).

Manipulations of genetic sequences were accomplished using the suite of programs available from the Genetics Computer Group Inc. (Wisconsin Package Version 9.0, Genetics Computer Group (GCG), Madison, WI). The GCG program "Pileup" was used with the gap creation default value of 12, and the gap extension default value of 4. The GCG "Gap" or "Bestfit" programs were used with the default gap creation penalty of 50 and the default gap extension penalty of 3. Unless otherwise stated, in all other cases GCG program default parameters were used.

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).

EXAMPLE 1

Isolation Of A Portion Of The *Yarrowia lipolytica* GPD

5 The present Example describes the identification of a portion of the *Yarrowia lipolytica* gene encoding GPD (SEQ ID NOs:11 and 12), by use of primers derived from conserved regions of other GPD sequences.

A comparison of the various protein sequences encoding GPD genes from *Saccharomyces cerevisiae* (GenBank Accession No. CAA24607; SEQ ID NO:1),
 10 *Schizosaccharomyces pombe* (GenBank Accession No. NP_595236; SEQ ID NO:2), *Aspergillus oryzae* (GenBank Accession No. AAK08065; SEQ ID NO:3), *Paralichthys olivaceus* (GenBank Accession No. BAA88638; SEQ ID NO:4), *Xenopus laevis* (GenBank Accession No. P51469; SEQ ID NO:5), and *Gallus gallus* (GenBank Accession No. DECHG3; SEQ ID NO:6) showed that there
 15 were several stretches of conserved amino acid sequence between the 6 different organisms (Figures 1A and 1B). Thus, two degenerate oligonucleotides (shown below), corresponding to the conserved 'KYDSTHG' (SEQ ID NO:7) and "TGAAKAV" (SEQ ID NO:8) amino acid sequences, respectively, were designed and used to amplify a portion of the coding region of GPD from *Y. lipolytica*:

20 Degenerated oligonucleotide YL193 (SEQ ID NO:9)

AAGTACGAYTCBACYCAYGG

Degenerated oligonucleotide YL194 (SEQ ID NO:10)

ACRGCCTTRGCRGCDCCRG

25 [Note: The nucleic acid degeneracy code used for SEQ ID NOs:9 and 10 was as follows: R= A/G; Y=C/T; B=C/G/T; and D=A/G/T.]

Based on the full-length sequences of the GPD sequences of Figure 1, it was hypothesized that the *Yarrowia lipolytica* GPD gene amplified as described above would be missing ~50 amino acids from its N- terminus and about ~115
 30 amino acids from its C-terminus.

The PCR amplification was carried out in a 50 µl total volume comprising: PCR buffer (containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide triphosphate, 10 pmole of each primer, 50 ng
 35 genomic DNA of *Y. lipolytica* (ATCC #76982) and 1 µl of Taq DNA polymerase (Epicentre Technologies). The thermocycler conditions were set for 35 cycles at 95°C for 1 min, 56°C for 30 sec, and 72°C for 1 min, followed by a final extension at 72°C for 10 min.

The PCR products were purified using a Qiagen PCR purification kit (Valencia, CA), and then further purified following gel electrophoresis in 1% (w/v) agarose. Subsequently, the PCR products were cloned into the pGEM-T-easy vector (Promega, Madison, WI). The ligated DNA was used
5 to transform cells of *E. coli* DH5 α and transformants were selected on LB agar containing ampicillin (100 μ g/mL). Analysis of the plasmid DNA from one transformant confirmed the presence of a plasmid of the expected size, and designated as "pT-GPD".

Sequence analyses showed that pT-GPD contained a 507 bp fragment
10 (SEQ ID NO:11). Identity of this sequence was determined by conducting BLAST (Basic Local Alignment Search Tool; Altschul, S. F., et al., *J. Mol. Biol.* 215:403-410 (1993)) searches for similarity to sequences contained in the BLAST "nr" database (comprising all non-redundant GenBank CDS translations, sequences derived from the 3-dimensional structure Brookhaven Protein Data
15 Bank, the SWISS-PROT protein sequence database, EMBL, and DDBJ databases). Similarity to all publicly available DNA sequences contained in the "nr" database was determined using the BLASTN algorithm provided by the National Center for Biotechnology Information (NCBI). The DNA sequence was translated in all reading frames and compared for similarity to all publicly
20 available protein sequences contained in the "nr" database, using the BLASTX algorithm (Gish, W. and States, D. J. *Nature Genetics* 3:266-272 (1993)) provided by the NCBI. The results of the BLAST comparison summarizing the sequence to which SEQ ID NO:11 has the most similarity are reported according to the % identity, % similarity, and Expectation value. "% Identity" is defined as
25 the percentage of amino acids that are identical between the two proteins. "% Similarity" is defined as the percentage of amino acids that are identical or conserved between the two proteins. "Expectation value" estimates the statistical significance of the match, specifying the number of matches, with a given score, that are expected in a search of a database of this size absolutely
30 by chance.

The 507 bp of pT-GPD was found to encode 169 amino acids (SEQ ID NO:12). This amino acid fragment had 77% identity and 84% similarity (Figure 2) with the GPD protein sequence of fission yeast (GenBank Accession No. NP_595236), with an expectation value of 6e-68. The *Yarrowia* sequence
35 possessed the 'KYDSTHG' (SEQ ID NO:7) and 'TGA AKAV' (SEQ ID NO:8) amino acid sequences (corresponding to the degenerate primers used to amplify the fragment) at its N- and C-termini. Further sequence comparison of this

partial GPD sequence determined that it also shared about 72% and 74% identity with the GPD proteins from chick (GenBank Accession No. DECHG3) and frog (GenBank Accession No. P51469), respectively (Figure 2).

EXAMPLE 2

5 Identification Of The *Yarrowia lipolytica* GPM

The present Example describes the identification of the *Yarrowia lipolytica* gene encoding GPM, by use of a *S. cerevisiae* GPM protein sequence as a query sequence against a *Y. lipolytica* genomic database.

Specifically, the *S. cerevisiae* GPM protein sequence (GenBank
10 Accession No. NP_012770; SEQ ID NO:13) was used in BLAST searches (as described in Example 1) against the public *Y. lipolytica* database of the "Yeast project *Genolevures*" (Center for Bioinformatics, LaBRI, Talence Cedex, France).

One contig ("Contig 2217"; SEQ ID NO:14) was identified that
15 encoded GPM in *Y. lipolytica*. Contig 2217 is 1049 bp in length, although 5 nucleotide positions had ambiguous sequence (having an "n" at nucleotide position 1020, "y" at positions 39, 62, 331; and a "m" at position 107). The DNA sequence of Contig 2217 was translated in all reading frames and compared for similarity to all publicly available protein
20 sequences contained in the "nr" database using the BLASTX algorithm (as described in Example 1). Based on these DNA and protein sequence analyses, it was determined that:

- The GPM translation initiation codon 'ATG' was at bp 388 within
25 SEQ ID NO:14; thus, Contig 2217 possessed about 388 bp upstream sequence relative to the 'ATG' codon; and
- Contig 2217 was missing one base at nucleotide position 470, which resulted in a frame shift.

The deduced coding region sequence of GPM that corresponded to Contig 2217 was 651 bp in length (SEQ ID NO:15) and the protein sequence was
30 encoded by SEQ ID NO:16. This 216 amino acid protein had 71% identity, 82% similarity, and an expectation value of 3e-81 with the GPM protein sequence of *S. cerevisiae* (GenBank Accession No. NP_012770; Goffeau, A., et al., *Science* 274(5287): 546 (1996)) (Figure 3).

EXAMPLE 3

35 Isolation Of The 5' Upstream Regions Of The *gpd* And *gpm* Genes From *Yarrowia lipolytica*

To isolate the GPD and GPM promoter regions from the genes identified in Examples 1 and 2, a genome-walking technique (TOPO[®] Walker Kit, Invitrogen, CA) was utilized.

Briefly, genomic DNA of *Y. lipolytica* was digested with *KpnI*, *SacI*, *SphI* or
5 *PacI*, and dephosphorylated with Calf Intestinal Alkaline Phosphatase (CIP),
separately. Primer extension reactions were then carried out individually using
the dephosphorylated DNA as template and one of the following oligonucleotides
as primer: YL206 (SEQ ID NO:17) for GPD and YL196 (SEQ ID NO:18) for
GPM. The primer extended products were linked with TOPO[®] linker and used as
10 templates for the first PCR reactions using primers of LinkAmp Primer1 and a
second appropriate oligonucleotide. Specifically, YL207 (SEQ ID NO:19) was
used as the second primer targeted for the upstream promoter region of GPD
and YL197 (SEQ ID NO:20) was used as the second primer for PCR reactions
targeted to the upstream GPM promoter region. The PCR amplifications were
15 carried out in a 50 µl total volume, comprising: PCR buffer (containing 10 mM
KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-
100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide
triphosphate, 10 pmole of each primer and 1 µl of Taq DNA polymerase
(Epicentre Technologies). The thermocycler conditions were set for 35 cycles at
20 95°C for 1 min, 56°C for 30 sec, and 72°C for 1 min, followed by a final extension
at 72°C for 10 min.

Second PCR reactions were then carried out using the first PCR product
as template and primers of LinkAmp primer 2 and the appropriate
oligonucleotide. Specifically, the first PCR product for GPD was used as
25 template in a reaction comprising LinkAmp primer 2 and YL208 (SEQ ID NO:21);
in contrast, the first PCR product for GPM was used as template in a reaction
comprising LinkAmp primer 2 and YL198 (SEQ ID NO:22). The PCR
amplifications were carried out as described above.

The PCR products comprising the 5' upstream regions of the GPD
30 and GPM genes were each individually purified using a Qiagen PCR
purification kit, followed by gel electrophoresis in 1% (w/v) agarose.
Products were then cloned into the pGEM-T-easy vector (Promega,
Madison, WI). The ligated DNA was used to transform *E. coli* DH5α and
transformants were selected on LB agar containing ampicillin (100 µg/mL).

35 Analysis of the plasmid DNA from one transformant comprising the
5' upstream region of the *gpd* gene confirmed the presence of the
expected plasmid, designated "pT-GPDP". Sequence analyses showed

that pT-GPDP contained a fragment of 1848 bp (SEQ ID NO:23), which included 1525 bp of 5' upstream sequence from the nucleotide 'A' (designated as +1) of the translation initiation codon 'ATG' of the GPD gene. A complete assembly of overlapping SEQ ID NOs:23 and 11
 5 yielded a single contig comprising 1525 bp upstream of the GPD initiation codon and 791 bp of the gene (SEQ ID NO:24; Figure 4). Further analysis of the partial gene sequence (+1 to +791) revealed the presence of an intron (base pairs +49 to +194). Thus, the partial cDNA sequence encoding the GPD gene in *Y. lipolytica* is only 645 bp in length (SEQ ID
 10 NO:25) and the corresponding protein sequence (SEQ ID NO:26) is 215 amino acids. The protein was compared via BLAST analysis for similarity to all publicly available protein sequences (as described in Example 1). Based on this analysis, it was determined that the partial GPD protein was most similar to the GPD of *Cryptococcus cyrvatus* (GenBank Accession
 15 No's Q9Y796 and AAD25080) (81% identical).

Analysis of the plasmid DNA from one transformant comprising the the 5' upstream region of the *gpm* gene confirmed the presence of the expected plasmid, designated "pT-GPML". Sequence analyses showed that pT-GPML contained a fragment of 953 bp (SEQ ID NO:27). This
 20 clone possessed 875 bp of 5' upstream sequence from the translation initiation codon of the GPM gene. Assembly of DNA corresponding to overlapping SEQ ID NOs:27 and 15 yielded a single contig of DNA represented as SEQ ID NO:28 (Figure 5). This contig therefore contained the -875 to +662 region of the GPM gene, wherein the 'A' position of the
 25 'ATG' translation initiation codon was designated as +1.

EXAMPLE 4

Synthesis Of pY5-30

The present Example describes the synthesis of pY5-30, comprising a TEF::GUS::XPR chimeric gene. This was required for comparative studies
 30 investigating the promoter activity of TEF, GPD and GPM, wherein constructs comprising each promoter and a reporter gene were prepared and analyzed (Examples 5-7). Specifically, the reporter was the *E. coli* gene encoding β -glucuronidase (GUS; Jefferson, R.A. *Nature*. 342(6251):837-838 (1989)).

Amplification Of The GUS Coding Region

35 The GUS coding region was amplified using pBI101 (Jefferson, R. A et al., *EMBO J.* 6:3901-3907 (1987)) as template and oligonucleotides YL33 (SEQ ID NO:29) and YL34 (SEQ ID NO:30) as primers. The PCR amplification was

carried out in a 50 µl total volume comprising: PCR buffer (containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide triphosphate, 10 pmole of each primer and 1 µl of Pfu DNA polymerase
 5 (Stratagene, San Diego, CA). The thermocycler conditions were set for 35 cycles at 95°C for 1 min, 56°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 10 min. The PCR products were digested with *NcoI* and *PacI*.

Synthesis Of Plasmid pY5-10

10 The plasmid pY5, a derivative of pINA532 (a gift from Dr. Claude Gaillardin, Insitut National Agronomics, Centre de biotechnologie Agro-Industrielle, laboratoire de Genetique Moleculaire et Cellularie INRA-CNRS, F-78850 Thiverval-Grignon, France), was constructed for expression of heterologous genes in *Yarrowia lipolytica*, as diagrammed in
 15 Figure 6. The partially-digested 3598 bp *EcoRI* fragment containing the ARS18 sequence and LEU2 gene of pINA532 was subcloned into the *EcoRI* site of pBluescript (Stratagene, San Diego, CA) to generate pY2.

The TEF promoter (Muller S., et al. *Yeast*, 14:1267-1283 (1998)) was amplified from *Y. lipolytica* genomic DNA by PCR using TEF5' (SEQ ID NO:31) and TEF3' (SEQ ID NO:32) as primers. PCR amplification was
 20 carried out in a 50 µl total volume containing: 100 ng *Yarrowia* genomic DNA, PCR buffer (containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide triphosphate, 10 pmole
 25 of each primer and 1 µl of PfuTurbo DNA polymerase (Stratagene, San Diego, CA). Amplification was carried out as follows: initial denaturation at 95 °C for 3 min, followed by 35 cycles of the following: 95 °C for 1 min, 56 °C for 30 sec, 72 °C for 1 min. A final extension cycle of 72 °C for 10 min was carried out, followed by reaction termination at 4 °C. The 418 bp PCR
 30 product was ligated into pCR-Blunt to generate pIP-tef. The *BamHI/EcoRV* fragment of pIP-tef was subcloned into the *BamHI/SmaI* sites of pY2 to generate pY4.

The XPR2 transcriptional terminator was amplified by PCR using pINA532 as template and XPR5' (SEQ ID NO:33) and XPR3' (SEQ ID
 35 NO:34) as primers. The PCR amplification was carried out in a 50 µl total volume, using the components and conditions described above. The 179 bp PCR product was digested with *SacII* and then ligated into the *SacII*

site of pY4 to generate pY5. Thus, pY5 (shown in Figure 6) contained: a *Yarrowia* autonomous replication sequence (ARS18); a ColE1 plasmid origin of replication; an ampicillin-resistance gene (Amp^R) for selection in *E. coli*; a *Yarrowia* LEU2 gene encoding isopropylmalate isomerase, for selection in *Yarrowia*; the translation elongation promoter ("TEF P"), for expression of heterologous genes in *Yarrowia*; and the extracellular protease gene terminator (XPR2) for transcriptional termination of heterologous gene expression in *Yarrowia*.

Plasmid pY5-10 (Figure 7A) was constructed as a derivative of pY5. First, pY5-4 (Figure 6) was constructed by three rounds of site-directed mutagenesis using pY5 as template. A *NcoI* site located inside the LEU2 reporter gene was eliminated from pY5 using oligonucleotides YL1 and YL2 (SEQ ID NOs:35 and 36) to generate pY5-1. A *NcoI* site was introduced into pY5-1 between the TEF promoter and XPR transcriptional terminator by site-directed mutagenesis using oligonucleotides YL3 and YL4 (SEQ ID NOs:37 and 38) to generate pY5-2. A *PacI* site was then introduced into pY5-2 between the TEF promoter and XPR transcriptional terminator using oligonucleotides YL23 and YL24 (SEQ ID NOs:39 and 40) to generate pY5-4. Finally, a *SaII* site was introduced into pY5-4 between the TEF promoter and the LEU2 gene by site-directed mutagenesis using oligonucleotides YL9 (SEQ ID NO:41) and YL10 (SEQ ID NO:42) as primers to generate pY5-10 (Figure 7A).

Synthesis Of Plasmid pY5-30

Plasmid pY5-30 (Figure 7B), comprising a TEF::GUS::XPR chimeric gene, was synthesized by inserting the *NcoI/PacI* PCR product comprising the GUS coding region (*supra*) into *NcoI/PacI* digested pY5-10.

EXAMPLE 5

Synthesis Of pYZGDG And pYZGMG

The present Example describes the synthesis of pYZGDG (comprising a GPD::GUS::XPR chimeric gene) and pYZGMG (comprising a GPM::GUS::XPR chimeric gene). Synthesis of these plasmids first required identification and amplification of the putative GPD and GPM promoter regions. Then, each putative promoter region was cloned into a derivative of pY5-30.

Identification And Amplification Of Putative Promoter Regions

After the isolation of the 5' upstream sequence of the GPD and GPM genes by genome walking, the translation start site was identified by looking for the consensus motif around the translation initiation 'ATG' codon and by

comparison of the translated coding region of the *Yarrowia* GPD and GPM genes with the GPD and GPM genes, respectively, from other organisms. The region upstream of the genes' 'ATG' start site was used to identify putative promoter regions.

5 Thus, the nucleotide region between the -968 position and the 'ATG' translation initiation site of the GPD gene (wherein the 'A' nucleotide of the 'ATG' translation initiation codon was designated as +1) was determined to contain the putative promoter region ("GPDPPro", provided as SEQ ID NO:43). In like manner, the nucleotide region between the -875 position and the 'ATG' translation initiation site of the GPM gene was determined to contain the putative promoter region ("GPMLPro", provided as SEQ ID NO:44).

 The putative promoter regions identified above were amplified by PCR. Specifically, GPDPPro was amplified with oligonucleotides YL211 (SEQ ID NO:45) and YL212 (SEQ ID NO:46) as primers and pT-GPDP (Example 3) as template. 15 GPMLPro was amplified with oligonucleotides YL203 (SEQ ID NO:47) and YL204 (SEQ ID NO:48) as primers and pT-GPML (Example 3) as template. The PCR amplifications were carried out in a 50 µl total volume, comprising: PCR buffer (containing 10 mM KCl, 10 mM (NH₄)₂SO₄, 20 mM Tris-HCl (pH 8.75), 2 mM MgSO₄, 0.1% Triton X-100), 100 µg/mL BSA (final concentration), 200 µM each deoxyribonucleotide triphosphate, 10 pmole of each primer and 1 µl of Pfu DNA polymerase (Stratagene, San Diego, CA). The thermocycler conditions were set for 35 cycles at 95°C for 1 min, 56°C for 30 sec, 72°C for 1 min, followed by a final extension at 72°C for 10 min.

 The PCR products were then purified using a Qiagen PCR purification kit and subjected to the following restriction digestions and ligation reactions: 25

- GPDPPro was completely digested with *Sall* and then partially digested with *NcoI*. The *Sall*/*NcoI* fragment was purified following gel electrophoresis in 1% (w/v) agarose and ligated to *NcoI*/*Sall* digested pY5-30 vector (Example 4) (wherein the *NcoI*/*Sall* digestion had excised the TEF promoter from the pY5-30 vector backbone). 30
- GPMLPro was digested with *NcoI* and *Sall* for 1 hr at 37°C and then purified following gel electrophoresis in 1% (w/v) agarose. The *NcoI*/*Sall*-digested PCR product was ligated to *NcoI*/*Sall* digested pY5-30 vector.

Ligated DNA from each reaction was then used to individually transform *E. coli* DH5α. Transformants were selected on LB agar containing ampicillin (100 µg/mL). 35

Analysis of the plasmid DNA from one transformant containing GPDPro confirmed the presence of the expected plasmid, designated "pYZGDG" (Figure 7C). Thus, this plasmid contained a chimeric gene comprising a GPD promoter, GUS reporter gene and XPR terminator.

5 Analysis of the plasmid DNA from one transformant containing GPMLPro confirmed the presence of the expected plasmid, designated "pYZGMG", and comprising a GPM::GUS::XPR chimeric gene (Figure 7D).

EXAMPLE 6

10 Transformation Of *Y. lipolytica* With pY5-30, pYZGDG And pYZGMG

The plasmids pY5-30 (Example 4; comprising a TEF::GUS::XPR chimeric gene), pYZGDG (Example 5; comprising a GPD::GUS::XPR chimeric gene) and pYZGMG (Example 5; comprising a GPM::GUS::XPR chimeric gene) were transformed separately into *Y. lipolytica* ATCC #76982 according to the method
15 of Chen, D. C. et al. (*Appl. Microbiol Biotechnol.* 48(2):232-235 (1997)).

Briefly, a leucine auxotroph of *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:

- 20 • 2.25 mL of 50% PEG, average MW 3350;
 • 0.125 mL of 2 M Li acetate, pH 6.0;
 • 0.125 mL of 2 M DTT; and
 • 50 µg sheared salmon sperm DNA.

About 500 ng of plasmid DNA was incubated in 100 µl of resuspended
25 cells, and maintained at 39 °C for 1 hr with vortex mixing at 15 min intervals. The cells were plated onto minimal media plates lacking leucine and maintained at 30 °C for 2 to 3 days.

Using this technique, transformants were obtained that contained pY5-30, pYZGDG and pYZGMG, respectively.

30 EXAMPLE 7

Comparative Analysis Of The TEF, GPD And GPM Promoter Activities In *Yarrowia lipolytica*

The activity of the TEF, GPD and GPM promoters were determined in *Yarrowia lipolytica* containing the pY5-30, pYZGDG and pYZGMG constructs,
35 each of which possessed a GUS reporter gene and an XPR terminator. GUS activity in each expressed construct was measured by histochemical and fluorometric assays (Jefferson, R. A. *Plant Mol. Biol. Reporter* 5:387-405 (1987)).

GUS Activities, Determined By Histochemical Assay

Specifically, two *Yarrowia lipolytica* strains containing plasmid pY5-30, two *Yarrowia lipolytica* strains containing plasmid pYZGDG and two *Yarrowia lipolytica* strains containing plasmid pYZGMG were each grown from single colonies in 3 mL minimal media (20 g/L glucose, 1.7 g/L yeast nitrogen base without amino acids, 1 g/L L-proline, 0.1 g/L L-adenine, 0.1 g/L L-lysine, pH 6.1) at 30 °C to an OD₆₀₀ ~1.0. Then, 100 µl of cells were collected by centrifugation, resuspended in 100 µl of histochemical staining buffer and incubated at 30 °C. [Staining buffer prepared by dissolving 5 mg of 5-bromo-4-chloro-3-indolyl glucuronide (X-Gluc) in 50 µl dimethyl formamide, followed by addition of 5 mL 50 mM NaPO₄, pH 7.0.]

The results of histochemical staining showed that the TEF promoter in construct pY5-30, the GPD promoter in construct pYZGDG and the GPM promoter in construct pYZGMG were all active. The GPD promoter appeared to be much stronger than the TEF promoter (Figure 8A), while the GPM promoter was at least as strong as the TEF promoter (Figure 8B).

GUS Activities, Determined By Fluorometric Assay

GUS activity was also assayed by fluorometric determination of the production of 4-methylumbelliferone from the corresponding substrate β-glucuronide (Jefferson, R. A., *supra*).

Yarrowia lipolytica strains containing plasmids pY5-30, pYZGDG and pYZGMG, respectively, were grown from single colonies in 3 mL minimal media (as described above) at 30 °C to an OD₆₀₀ ~1.0. Then, the 3 mL cultures were each added to a 500 mL flask containing 50 mL minimal media and grown in a shaking incubator at 30 °C for about 24 hrs. The cells were collected by centrifugation, resuspended in Promega Cell Lysis Buffer and lysed using the BIO 101 Biopulverizer system (Vista, CA). After centrifugation, the supernatants were removed and kept on ice.

For each fluorometric assay, 100 µl of extract was added to 700 µl of GUS assay buffer (2 mM 4-methylumbelliferyl-β-D-glucuronide ("MUG") in extraction buffer) and placed at 37 °C. Aliquots of 100 µl were taken at 0, 30 and 60 min time points and added to 900 µl of stop buffer (1 M Na₂CO₃). Each time point was read using a Fluorimeter (CytoFluorR Series 4000, Framingham, MA) set to an excitation wavelength of 360 nm and an emission wavelength of 455 nm. Total protein concentration of each sample was determined using 10 µl of extract and 200 µl of BioRad Bradford reagent (Bradford, M. M. *Anal. Biochem.* 72:248-

254 (1976)). GUS activity was expressed as nmoles of 4-MU per minute per mg of protein.

Results of these fluorometric assays are shown in Figure 9. Specifically, Figure 9A showed that the GPD promoter was 3 times stronger than the benchmark TEF promoter in *Y. lipolytica*; in contrast, Figure 9B showed that the GUS activity of the GPM promoter was about 110% as active as the benchmark TEF promoter.

EXAMPLE 8

Use of the GPD Promoter For $\Delta 15$ Desaturase Expression

10 In *Yarrowia lipolytica*

The present Example describes the construction of a chimeric gene comprising a GPD promoter, fungal $\Delta 15$ desaturase and the XPR terminator, and the expression of this gene in *Y. lipolytica*. Since transformed host cells were able to produce ALA (while wildtype *Y. lipolytica* do not possess any $\Delta 15$ desaturase activity), this confirms the ability of the GPD promoter to drive expression of heterologous PUFA biosynthetic pathway enzymes in oleaginous yeast cells such as *Y. lipolytica*.

20 Construction Of Plasmid pY34, Comprising A GPD::Fm1::XPR Chimeric Gene

First, plasmid pY5-13 was constructed as a derivative of pY5 (from Example 4). Specifically, pY5-13 was constructed by 6 rounds of site-directed mutagenesis using pY5 as template. Both *Sall* and *ClaI* sites were eliminated from pY5 by site-directed mutagenesis using oligonucleotides YL5 and YL6 (SEQ ID NOs:49 and 50) to generate pY5-5. A *Sall* site was introduced into pY5-5 between the LEU2 gene and the TEF promoter by site-directed mutagenesis using oligonucleotides YL9 and YL10 (SEQ ID NOs:41 and 42) to generate pY5-6. A *PacI* site was introduced into pY5-6 between the LEU2 gene and ARS18 using oligonucleotides YL7 and YL8 (SEQ ID NOs:51 and 52) to generate pY5-8. A *NcoI* site was introduced into pY5-8 around the translation start codon of the TEF promoter using oligonucleotides YL3 and YL4 (SEQ ID NOs:37 and 38) to generate pY5-9. The *NcoI* site inside the LEU2 gene of pY5-9 was eliminated using YL1 and YL2 oligonucleotides (SEQ ID NOs:35 and 36), to generate pY5-12. Finally, a *BsiWI* site was introduced into pY5-12 between the ColEI and XPR region using oligonucleotides YL61 and YL62 (SEQ ID NOs:53 and 54) to generate pY5-13.

A purified *Sall*/*NcoI* fragment comprising GPDP_{ro} (from Example 5) was ligated to *NcoI*/*Sall* digested pY5-13 vector (wherein the *NcoI*/*Sall* digestion had excised the TEF promoter from the pY5-13 vector backbone) to yield "pY5-13GPD". Thus, pY5-13GPD comprised a GPD promoter::XPR terminator expression cassette.

The *Nco I* site at the 3' end of the promoter fragment in pY5-13GPD was converted to a *Not I* site to yield "pY5-13GPDN". For this, the GPD promoter was re-amplified by PCR using GPD_{sense} (SEQ ID NO:55) and GPD_{antisense} (SEQ ID NO:56) primers with a *Not I* site. The resultant promoter fragment was digested with *Sal I* and *Not I* and cloned into the *Sal/NotI* site of pY5-13 (thus removing the TEF promoter) to produce pY5-13GPDN.

The ORF encoding the *Fusarium moniliforme* strain M-8114 $\Delta 15$ desaturase (SEQ ID NO:57; see co-pending U.S. Provisional Application No. 60/519191) was PCR amplified using the cDNA clone ffm1c.pK001.g23 (E.I. du Pont de Nemours and Co., Inc., Wilmington, DE) containing the full-length cDNA as the template and using upper and lower primers P192 (SEQ ID NO:59) and P193 (SEQ ID NO:60). The PCR was carried out in an Eppendorf Mastercycler Gradient Cycler using *Pfu* polymerase, per the manufacturer's recommendation. Amplification was carried out as follows: initial denaturation at 95 °C for 1 min, followed by 30 cycles of denaturation at 95 °C for 30 sec, annealing at 58 °C for 1 min, and elongation at 72 °C for 1 min. A final elongation cycle at 72 °C for 10 min was carried out, followed by reaction termination at 4 °C.

The correct-sized (ca. 1240 bp) fragment was obtained, purified from an agarose gel using a Qiagen DNA purification kit, digested with *Not I* and cloned into the *Not I* site between the GPD promoter and XPR terminator of plasmid pY5-13GPDN. This resulted in creation of plasmid "pY34", which contained a GPD::Fm1::XPR chimeric gene.

30 Expression of Plasmid pY34 (GPD::Fm1::XPR) in *Yarrowia lipolytica*

pY5 (vector alone control, from Example 4) and pY34 (GPDP::Fm1::XPR) were each individually transformed into wild type (WT) *Yarrowia lipolytica* ATCC #76892, using the transformation procedure described in Example 6, and selected on Bio101 DOB/CSM-Leu plates.

35 Single colonies of wild type and transformant cells were each grown in 3 mL minimal media (formulation/L: 20 g glucose, 1.7 g yeast nitrogen base, 1 g L-proline, 0.1 g L-adenine, 0.1 g L-lysine, pH 6.1) at 30

°C to an OD₆₀₀ ~ 1.0. The cells were harvested, washed in distilled water, speed vacuum dried and subjected to direct trans-esterification and GC analysis. Specifically, for fatty acid analysis cells were collected by centrifugation and lipids were extracted as described in Bligh, E. G. & Dyer, W. J. (*Can. J. Biochem. Physiol.* 37:911-917 (1959)). Fatty acid methyl esters were prepared by transesterification of the lipid extract with sodium methoxide (Roughan, G., and Nishida I. *Arch Biochem Biophys.* 276(1):38-46 (1990)) and subsequently analyzed with a Hewlett-Packard 6890 GC fitted with a 30-m X 0.25 mm (i.d.) HP-INNOWAX (Hewlett-Packard) column. The oven temperature was from 170 °C (25 min hold) to 185 °C at 3.5 °C/min.

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

The fatty acid profile of wildtype *Yarrowia* and each of the transformants are shown below in Table 2. Fatty acids are identified as 16:0 (palmitate), 16:1 (palmitoleic acid), 18:0, 18:1 (oleic acid), 18:2 (LA) and 18:3 (ALA) and the composition of each is presented as a % of the total fatty acids.

Table 2

Expression of *Fusarium* Δ15 Desaturase In *Yarrowia lipolytica*

<i>Y. lipolytica</i> strain	% 16:0	% 16:1	% 18:0	% 18:1	% 18:2	% ALA
WT	12	9	1	34	44	0
WT + GPD:Fm1:XPR	10	10	1	37	7	31

The results above demonstrated that the GPD promoter is suitable to drive expression of the Δ15 desaturase, leading to production of ALA in *Yarrowia*.

CL2299 PCT SEQ
SEQUENCE LISTING

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<120> GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE AND PHOSPHOGLYCERATE
MUTASE PROMOTERS FOR GENE EXPRESSION IN OLEAGINOUS YEAST

<130> CL2299 PCT

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<170> PatentIn version 3.2

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Gly Lys Leu Val Ile Asp Gly His Ser Ile Asp Val His Asn Glu Arg
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Lys Lys Asp Trp Arg Gly Gly Arg Gly Ala Ser Ala Asn Ile Ile Pro
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Page 4

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 Lys Leu Thr Gly Met Ala Phe Arg Val Pro Thr Pro Asn Val Ser Val
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<213> Yarrowia lipolytica

<400> 12

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20 25 30

Arg Asp Pro Ser Asn Ile Pro Trp Gly Lys Ala Gly Ala Asp Tyr Val
35 40 45

Val Glu Ser Thr Gly Val Phe Thr Gly Lys Glu Ala Ala Ser Ala His
50 55 60

Leu Lys Gly Gly Ala Lys Lys Val Ile Ile Ser Ala Pro Ser Gly Asp
65 70 75 80

Ala Pro Met Phe Val Val Gly Val Asn Leu Asp Ala Tyr Lys Pro Asp
85 90 95

Met Thr Val Ile Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro
100 105 110

Leu Ala Lys Val Val Asn Asp Lys Tyr Gly Ile Ile Glu Gly Leu Met
115 120 125

WO 2005/003310

PCT/US2004/020432

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Thr Thr Val His Ser Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro
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Ser His Lys Asp Trp Arg Gly Gly Arg Thr Ala Ser Gly Asn Ile Ile
 145 150 155 160

Pro Ser Ser Thr Gly Ala Ala Lys Ala
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<212> PRT

<213> Saccharomyces cerevisiae (GenBank Accesssion No. NP_012770)

<400> 13

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 20 25 30

Gln Gln Glu Ala Ala Arg Ala Gly Glu Leu Leu Lys Glu Lys Lys Val
 35 40 45

Tyr Pro Asp Val Leu Tyr Thr Ser Lys Leu Ser Arg Ala Ile Gln Thr
 50 55 60

Ala Asn Ile Ala Leu Glu Lys Ala Asp Arg Leu Trp Ile Pro Val Asn
 65 70 75 80

Arg Ser Trp Arg Leu Asn Glu Arg His Tyr Gly Asp Leu Gln Gly Lys
 85 90 95

Asp Lys Ala Glu Thr Leu Lys Lys Phe Gly Glu Glu Lys Phe Asn Thr
 100 105 110

Tyr Arg Arg Ser Phe Asp Val Pro Pro Pro Pro Ile Asp Ala Ser Ser
 115 120 125

Pro Phe Ser Gln Lys Gly Asp Glu Arg Tyr Lys Tyr Val Asp Pro Asn
 130 135 140

Val Leu Pro Glu Thr Glu Ser Leu Ala Leu Val Ile Asp Arg Leu Leu
 145 150 155 160

Pro Tyr Trp Gln Asp Val Ile Ala Lys Asp Leu Leu Ser Gly Lys Thr
 165 170 175

Val Met Ile Ala Ala His Gly Asn Ser Leu Arg Gly Leu Val Lys His
 180 185 190

Leu Glu Gly Ile Ser Asp Ala Asp Ile Ala Lys Leu Asn Ile Pro Thr

CL2299 PCT SEQ

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 <211> 216
 <212> PRT
 <213> Yarrowia lipolytica

<400> 16

Met Pro Lys Leu Ile Leu Leu Arg His Gly Gln Ser Asp Trp Asn Glu
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Lys Asn Leu Phe Thr Gly Trp Val Asp Val Lys Leu Ser Glu Leu Gly
20 25 30

His Thr Glu Ala Lys Arg Ala Gly Thr Leu Leu Lys Glu Ser Gly Leu
35 40 45

Lys Pro Gln Ile Leu Tyr Thr Ser Glu Leu Ser Arg Ala Ile Gln Thr
50 55 60

Ala Asn Ile Ala Leu Asp Glu Ala Asp Arg Leu Trp Ile Pro Thr Lys
65 70 75 80

Arg Ser Trp Arg Leu Asn Glu Arg His Tyr Gly Ala Leu Gln Gly Lys
85 90 95

Asp Lys Ala Ala Thr Leu Ala Glu Tyr Gly Pro Glu Gln Phe Gln Leu
100 105 110

Trp Arg Arg Ser Phe Asp Val Pro Pro Pro Pro Ile Ala Asp Asp Asp
115 120 125

CL2299 PCT SEQ

Lys Trp Ser Gln Tyr Asn Asp Glu Arg Tyr Gln Asp Ile Pro Lys Asp
 130 135 140

Ile Leu Pro Lys Thr Glu Ser Leu Lys Leu Val Ile Asp Arg Leu Leu
 145 150 155 160

Pro Tyr Tyr Asn Ser Asp Ile Val Pro Asp Leu Lys Ala Gly Lys Thr
 165 170 175

Val Leu Ile Ala Ala His Gly Asn Ser Leu Arg Ala Leu Val Lys His
 180 185 190

Leu Asp Gly Ile Ser Asp Asp Asp Ile Ala Ala Leu Asn Ile Pro Thr
 195 200 205

Gly Ile Pro Leu Val Leu Arg Pro
 210 215

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<400> 18
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25

<210> 19
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<220>
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CL2299 PCT SEQ

<223> Primer YL197

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<210> 21

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<210> 23

<211> 1848

<212> DNA

<213> *Yarrowia lipolytica*

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atgtacgatg ggtgcctcaa ttgtgcgata ctactacaag tgcagcacgc tcgtgcccgt 180

accctacttt gtcggacgtc cctgctccct cgttcaacat ctcaagctca acaatcagtg 240

ttggacactg caacgctagc agccggtacg tggcttttagc cccatgctcc atgctccatg 300

ctccatgctc tgggcctatg agctagccgt ttggcgcaca tagcatagtg acatgtcgat 360

caagtcaaag tcgaggtgtg gaaaacgggc tgcgggtcgc caggggcctc acaagcgcct 420

ccaccgcaga cgcccacctc gttagcgtcc attgcgatcg tctcgggtaca tttgggttaca 480

ttttgcgaca gggttgaaatg aatcggccga cgctcggtag tcggaaagag ccgggaccgg 540

ccggcgagca taaaccggac gcagtaggat gtcctgcacg ggtctttttg tgggggtgtgg 600

agaaaggggt gcttggagat ggaagccggt agaaccgggc tgcttgtgct tggagatgga 660

agccggtaga accgggctgc ttgggggggat ttggggccgc tgggctccaa agaggggtag 720

gcatttcggtt ggggttacgt aattgcggca tttgggtcct gcgcgcatgt cccattgggtc 780

agaattagtc cggataggag acttatcagc caatcacagc gccggatcca cctgtagggt 840

gggttggggtg ggagcacccc tccacagagt agagtcaaac agcagcagca acatgatagt 900

tgggggtgtg cgtgttaaag gaaaaaaaaaag aagcttgggt tatattcccg ctctatttag 960

CL2299 PCT SEQ

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 <212> DNA
 <213> *Yarrowia lipolytica*

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CL2299 PCT SEQ

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<212> DNA
<213> Yarrowia lipolytica

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tacgctgctt acatgttcaa gtacgactcc acccacggcc gattcaaggg caaggctcag 180
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CL2299 PCT SEQ

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<210> 26
<211> 215
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<213> Yarrowia lipolytica

<400> 26

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20 25 30

Asn Asp Pro Phe Ile Asp Thr Glu Tyr Ala Ala Tyr Met Phe Lys Tyr
35 40 45

Asp Ser Thr His Gly Arg Phe Lys Gly Lys Val Glu Ala Lys Asp Gly
50 55 60

Gly Leu Ile Ile Asp Gly Lys His Ile Gln Val Phe Gly Glu Arg Asp
65 70 75 80

Pro Ser Asn Ile Pro Trp Gly Lys Ala Gly Ala Asp Tyr Val Val Glu
85 90 95

Ser Thr Gly Val Phe Thr Gly Lys Glu Ala Ala Ser Ala His Leu Lys
100 105 110

Gly Gly Ala Lys Lys Val Ile Ile Ser Ala Pro Ser Gly Asp Ala Pro
115 120 125

Met Phe Val Val Gly Val Asn Leu Asp Ala Tyr Lys Pro Asp Met Thr
130 135 140

Val Ile Ser Asn Ala Ser Cys Thr Thr Asn Cys Leu Ala Pro Leu Ala
145 150 155 160

Lys Val Val Asn Asp Lys Tyr Gly Ile Ile Glu Gly Leu Met Thr Thr
165 170 175

Val His Ser Ile Thr Ala Thr Gln Lys Thr Val Asp Gly Pro Ser His
180 185 190

Lys Asp Trp Arg Gly Gly Arg Thr Ala Ser Gly Asn Ile Ile Pro Ser
195 200 205

CL2299 PCT SEQ

Ser Thr Gly Ala Ala Lys Ala
210 215

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<213> Yarrowia lipolytica

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<211> 1537
<212> DNA
<213> Yarrowia lipolytica

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<222> (1507)..(1507)
<223> n is a, c, g, or t

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gttgcatccg ttgagagcgg tttgttttta accttctcca tgtgctcact cagggttttgg 180
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actgcctcga tgccctaacc ttgccccaaa ataagacaac tgtcctcggt taagcgcaac 360
cctattcagc gtcacgtcat aatagcggtt ggatagcact agtctatgag gagcgtttta 420

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<220>
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<210> 30
 <211> 36
 <212> DNA
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<220>
 <223> Primer YL34

<400> 30
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<210> 31
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CL2299 PCT SEQ

<220>
 <223> Primer TEF5'

 <400> 31
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 <220>
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 <210> 33
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 <220>
 <223> Primer XPR5'

 <400> 33
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 <210> 34
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 <220>
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 <220>
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 <210> 36
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 <400> 36
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CL2299 PCT SEQ

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<220>
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<220>
<223> Primer YL4

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<210> 39
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<212> DNA
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<220>
<223> Primer YL23

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<210> 40
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<220>
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<210> 41
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<220>
<223> Primer YL9

<400> 41
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<210> 42
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<220>
<223> Primer YL10

CL2299 PCT SEQ

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<210> 43
<211> 971
<212> DNA
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 35 40 45

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Cys Thr Gly Val Trp Ile Leu Gly His Glu Cys Gly His Gly Ala Phe
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Ser Leu His Gly Lys Val Asn Asn Val Thr Gly Trp Phe Leu His Ser
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His Arg Phe Thr Gly His Met Asp Leu Asp Met Ala Phe Val Pro Lys
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Thr Glu Pro Lys Pro Ser Lys Ser Leu Met Ile Ala Gly Ile Asp Val
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Ala Glu Leu Val Glu Asp Thr Pro Ala Ala Gln Met Val Lys Leu Ile
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Phe His Gln Leu Phe Gly Trp Gln Ala Tyr Leu Phe Phe Asn Ala Ser
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Ser Gly Lys Gly Ser Lys Gln Trp Glu Pro Lys Thr Gly Leu Ser Lys
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Trp Phe Arg Val Ser His Phe Glu Pro Thr Ser Ala Val Phe Arg Pro
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CL2299 PCT SEQ

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Ala Ile Thr Tyr Leu His His His His Thr Glu Leu Pro His Tyr Thr
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Glu Phe Gly Phe Ile Gly Lys His Leu Phe His Gly Ile Ile Glu Lys
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34

CLAIMS

What is claimed is:

1. A method for the expression of a coding region of interest in
5 a transformed yeast cell comprising:
 - a) providing a transformed yeast cell having a chimeric gene comprising:
 - (i) a promoter region of a *Yarrowia* gene selected from the group consisting of: a *gpm* gene and a *gpd* gene; and,
 - 10 (ii) a coding region of interest expressible in the yeast cell;wherein the promoter region is operably linked to the coding region of interest; and
 - b) growing the transformed yeast cell of step (a) under conditions whereby the chimeric gene of step (a) is
15 expressed.
2. A method according to Claim 1 wherein the *Yarrowia* gene is isolated from *Yarrowia lipolytica*.
- 20 3. A method according to Claim 1 wherein the promoter region of the *gpm* gene is contained within a nucleic acid fragment selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:44.
4. A method according to Claim 1 wherein the promoter region
25 of the *gpd* gene is contained within a nucleic acid fragment selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24 and SEQ ID NO:43.
5. A method according to either Claim 3 or 4 wherein the promoter region contains at least one mutation that does not diminish its
30 promoter activity.
6. A method according to Claim 5 wherein the promoter activity is at least about 20% to at least about 400% of the promoter activity of the wildtype promoter activity.
35
7. A method according to Claim 1 wherein the transformed yeast cell is an oleaginous yeast.

8. A method of Claim 7, wherein the oleaginous yeast is a member of a genus selected from the group consisting of *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

5

9. A method according to Claim 8, wherein the oleaginous yeast is selected from the group consisting of *Yarrowia lipolytica* ATCC #20362, *Yarrowia lipolytica* ATCC #8862, *Yarrowia lipolytica* ATCC #18944, *Yarrowia lipolytica* ATCC #76982 and *Yarrowia lipolytica* LGAM S(7)1.

10

10. A method according to Claim 1 wherein the coding region of interest encodes a polypeptide selected from the group consisting of: desaturases, elongases, aminopeptidases, amylases, carbohydrases, carboxypeptidases, catalyases, cellulases, chitinases, cutinases, cyclodextrin glycosyltransferases, deoxyribonucleases, esterases, α -galactosidases, β -galactosidases, glucoamylases, α -glucosidases, β -glucanases, β -glucosidases, invertases, laccases, lipases, mannosidases, mutanases, oxidases, pectinolytic enzymes, peroxidases, phospholipases, phytases, polyphenoloxidases, proteolytic enzymes, ribonucleases, transglutaminases and xylanases.

15

20

11. A method for the production of an ω -3 or an ω -6 fatty acid comprising:

25

(a) providing a transformed oleaginous yeast comprising a chimeric gene, comprising:

(i) a promoter region of a *Yarrowia* gene selected from the group consisting of: a *gpm* gene and a *gpd* gene; and

(ii) a coding region encoding at least one enzyme of the ω -3/ ω -6 fatty acid biosynthetic pathway;

30

wherein the promoter region and coding region are operably linked; and

(b) contacting the transformed oleaginous yeast of step (a) under conditions whereby the at least one enzyme of the ω -3/ ω -6 fatty acid biosynthetic pathway is expressed and a ω -3 or ω -6 fatty acid is produced; and

35

(c) optionally recovering the ω -3 or ω -6 fatty acid.

12. A method according to Claim 11 wherein the *Yarrowia* gene is isolated from *Yarrowia lipolytica*.

5 13. A method according to Claim 11 wherein the promoter region of the *gpm* gene is contained within a nucleic acid fragment selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:44.

10 14. A method according to Claim 11 wherein the promoter region of the *gpd* gene is contained within a nucleic acid fragment selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24 and SEQ ID NO:43.

15 15. A method according to Claim 11 wherein the coding region of interest encodes a polypeptide selected from the group consisting of: desaturases and elongases.

20 16. A method according to Claim 15 wherein the desaturase is selected from the group consisting of: $\Delta 9$ desaturase, $\Delta 12$ desaturase, $\Delta 6$ desaturase, $\Delta 5$ desaturase, $\Delta 17$ desaturase, $\Delta 15$ desaturase and $\Delta 4$ desaturase.

25 17. A method according to Claim 11 wherein the oleaginous yeast is a member of a genus selected from the group of consisting of: *Yarrowia*, *Candida*, *Rhodotorula*, *Rhodospiridium*, *Cryptococcus*, *Trichosporon* and *Lipomyces*.

18. A method according to Claim 17 wherein the oleaginous yeast is *Yarrowia lipolytica*.

30 19. A method according to Claim 18, wherein the *Yarrowia lipolytica* is a strain selected from the group consisting of *Yarrowia lipolytica* ATCC #20362, *Yarrowia lipolytica* ATCC #8862, *Yarrowia lipolytica* ATCC #18944, *Yarrowia lipolytica* ATCC #76982 and *Yarrowia lipolytica* LGAM S(7)1.

35

20. A method according to Claim 11 wherein the ω -3 or ω -6 fatty acid is selected from the group consisting of: linoleic acid, α -linolenic acid,

γ -linolenic acid, stearidonic acid, dihomogamma-linoleic acid, eicosatetraenoic acid, arachidonic acid, eicosapentaenoic acid, docosapentaenoic acid and docosahexaenoic acid.

5 21. An isolated nucleic acid molecule comprising a *gpd* promoter selected from the group consisting of SEQ ID NO:23, SEQ ID NO:24 and SEQ ID NO:43.

10 22. An isolated nucleic acid molecule comprising a *gpd* promoter selected from the group consisting of SEQ ID NO:27, SEQ ID NO:28 and SEQ ID NO:44.

Figure 1A

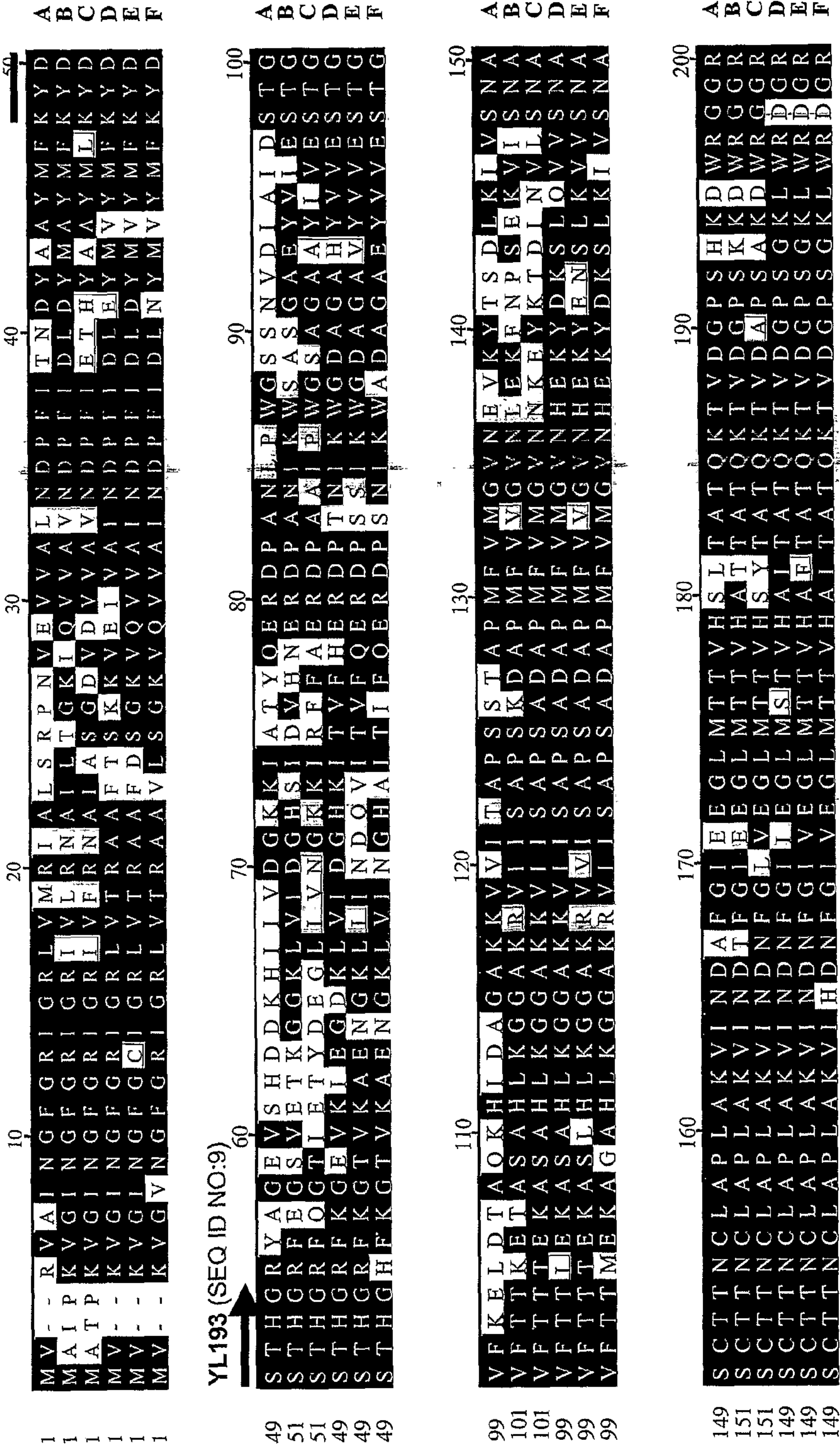
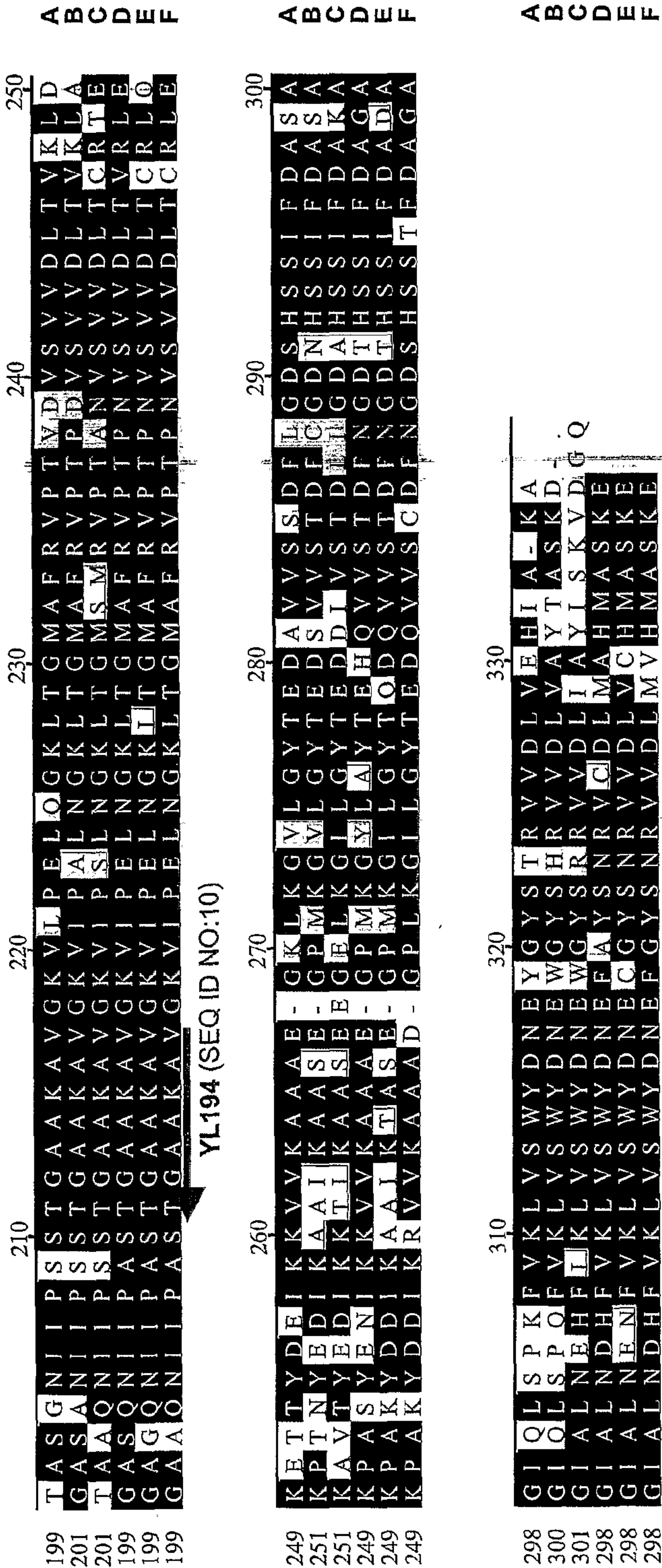
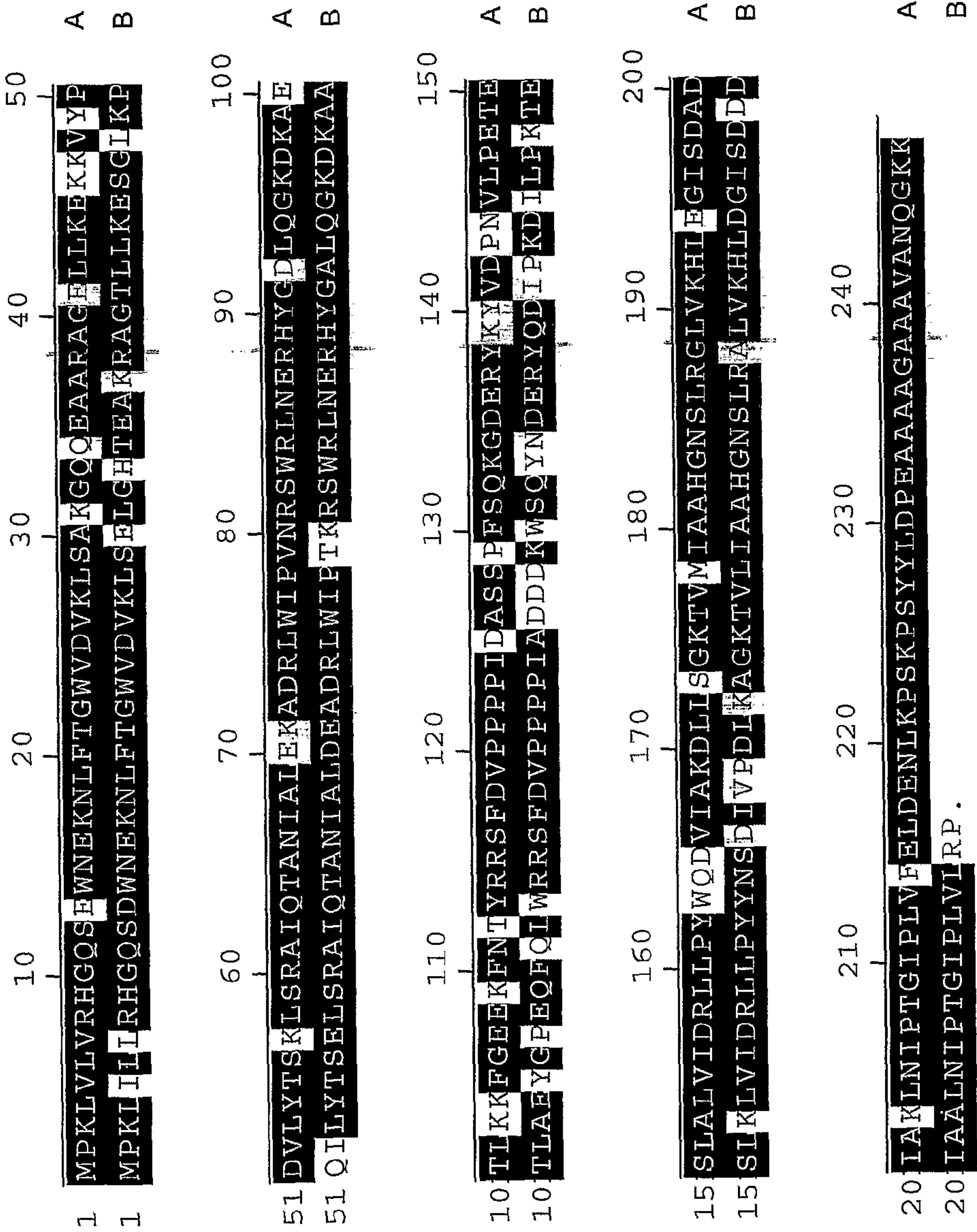


Figure 1B



- A: *Saccharomyces cerevisiae* (SEQ ID NO:1; GenBank Accession No. CAA24607)
- B: *Schizosaccharomyces pombe* (SEQ ID NO:2; GenBank Accession No. NP_595236)
- C: *Aspergillus oryzae* (SEQ ID NO:3; GenBank Accession No. AAK08065)
- D: *Paralichthys olivaceus* (SEQ ID NO:4; GenBank Accession No. BAA88638)
- E: *Xenopus laevis* (SEQ ID NO:5; GenBank Accession No. P51469)
- F: *Gallus gallus* (SEQ ID NO:6; GenBank Accession No. DECHG3)

Figure 3



A: *Saccharomyces cerevisiae* (SEQ ID NO:13; GenBank Accession No. NP_012770)
B: *Yarrowia lipolytica* (SEQ ID NO:16)

Figure 4
2316 bp encoding GPD

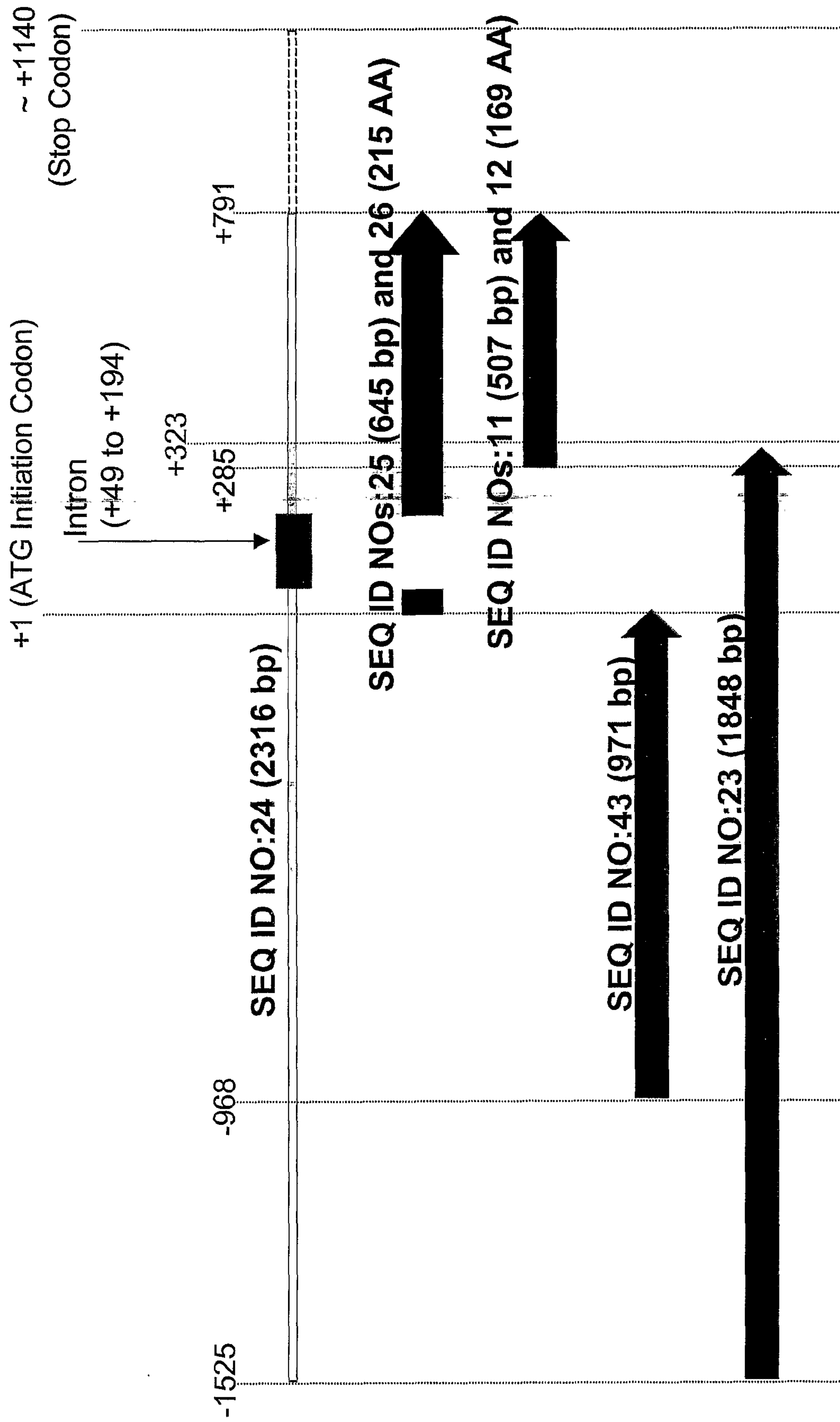


Figure 5
1537 bp encoding GPM

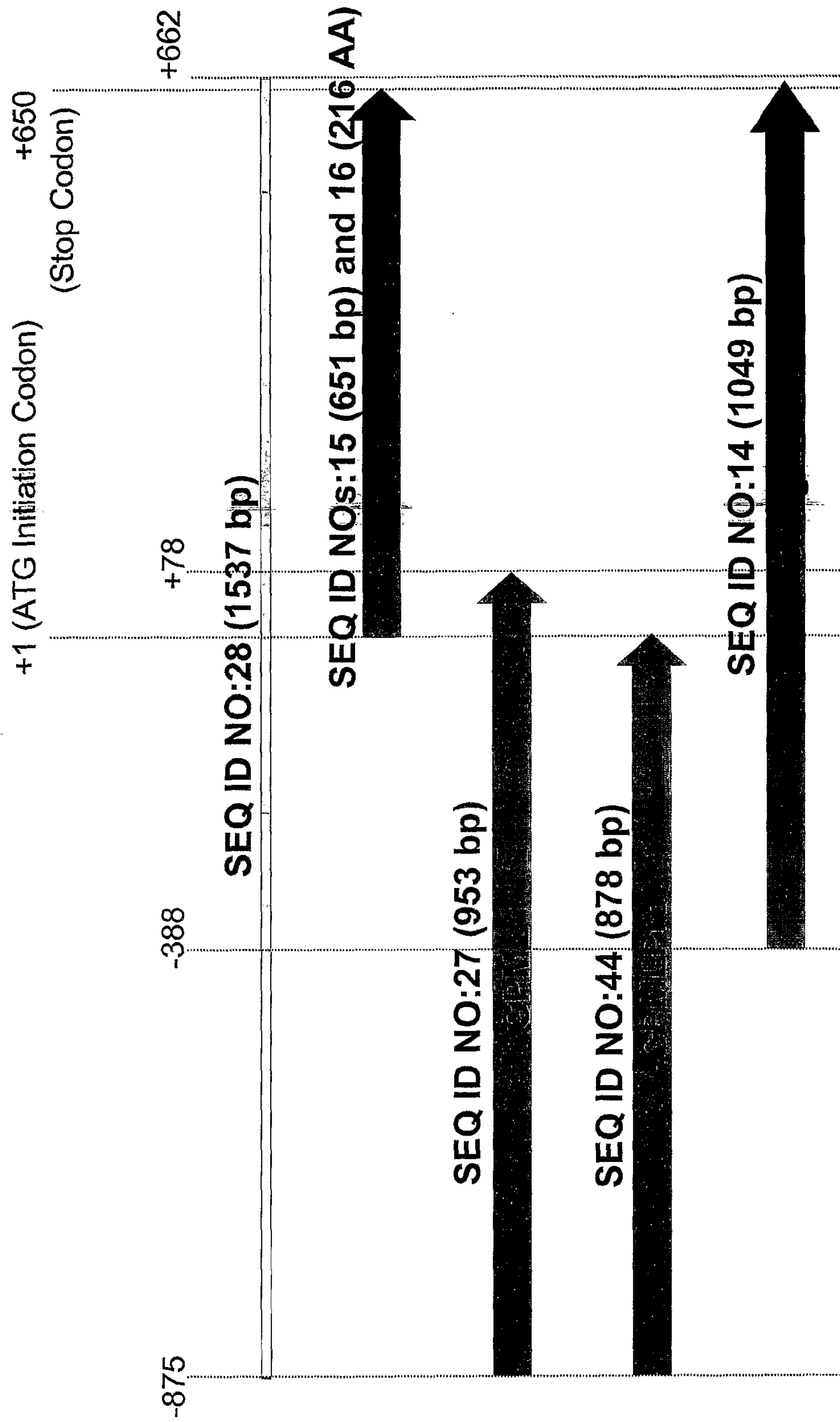


Figure 6

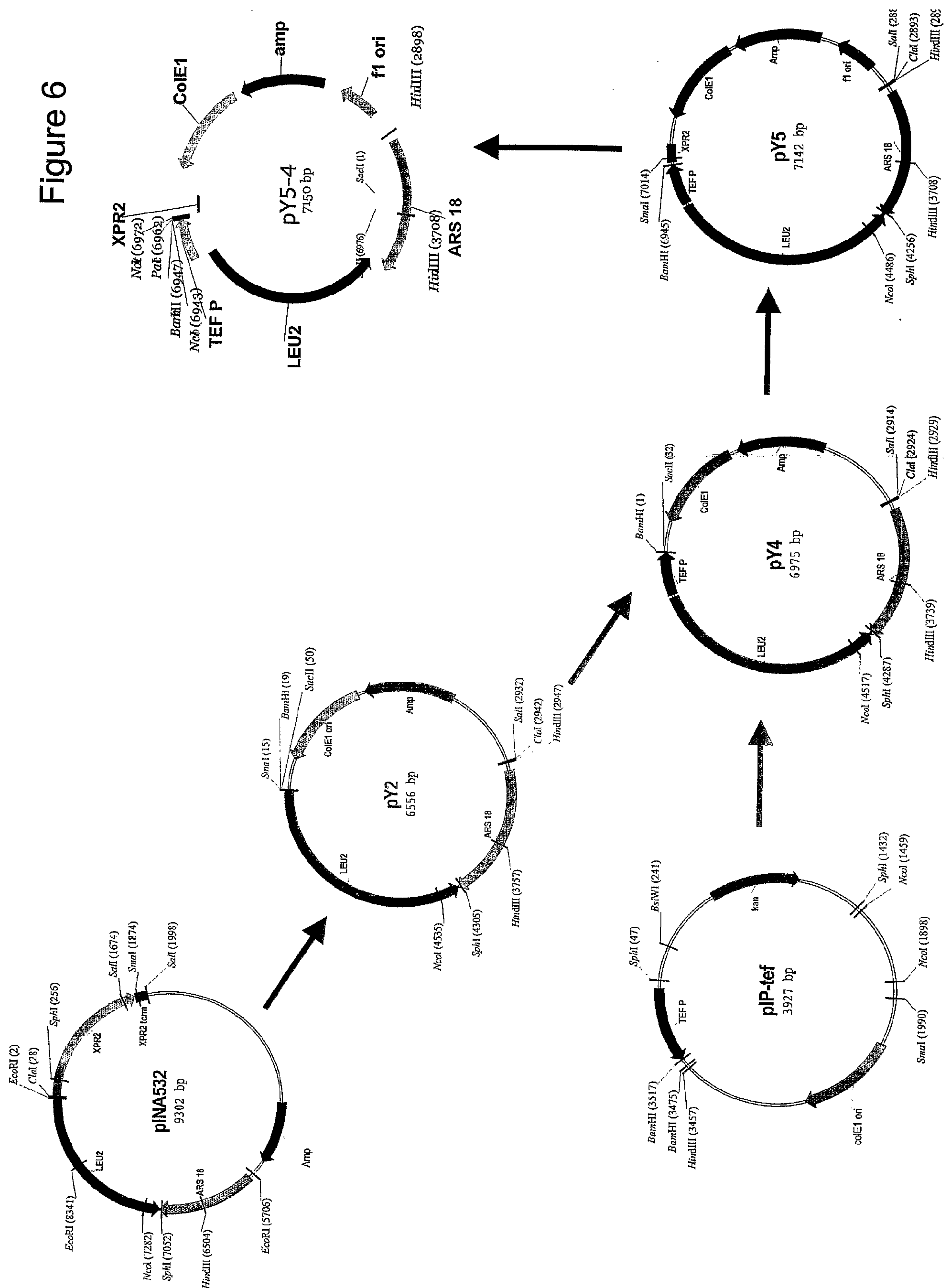


Figure 7

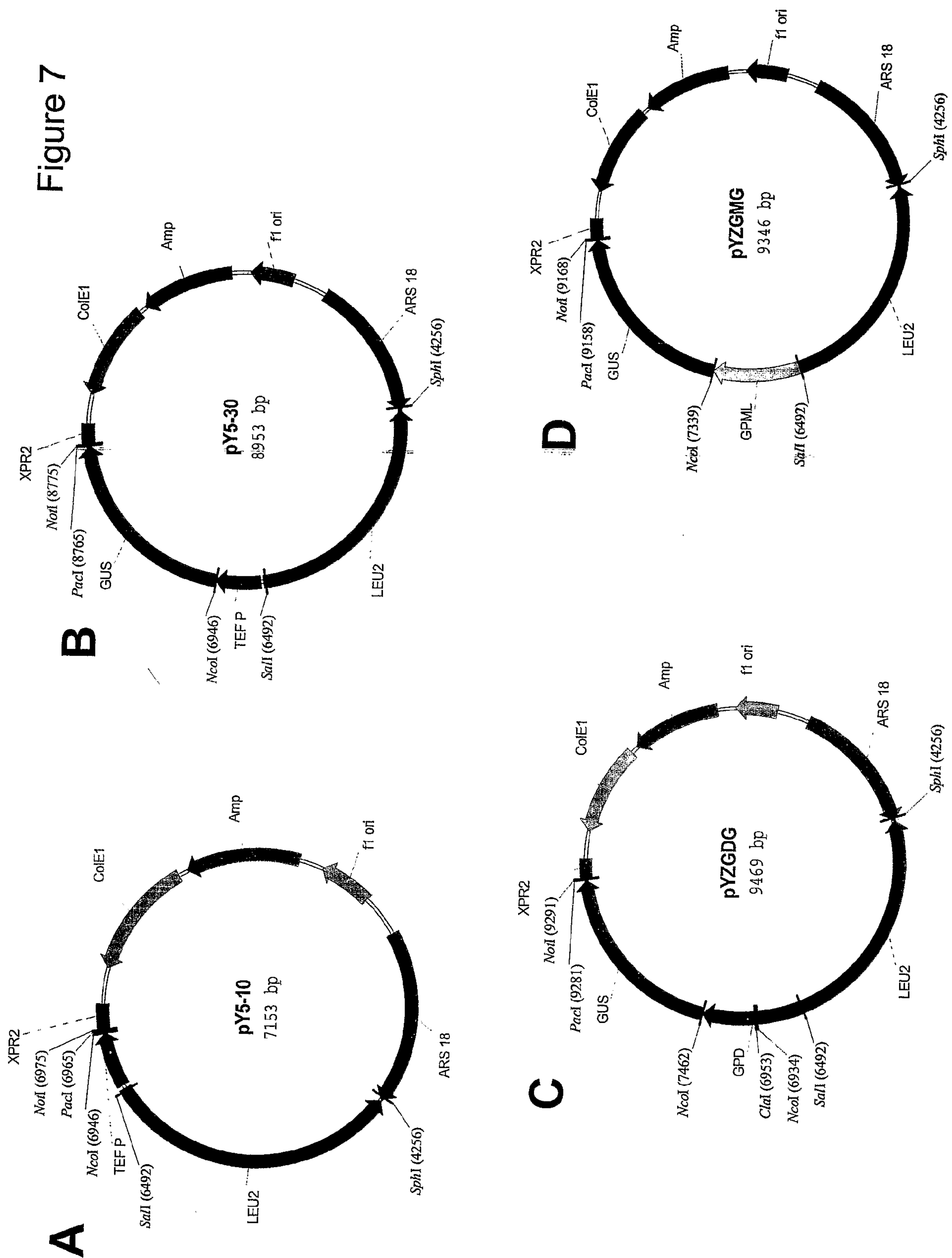


Figure 8

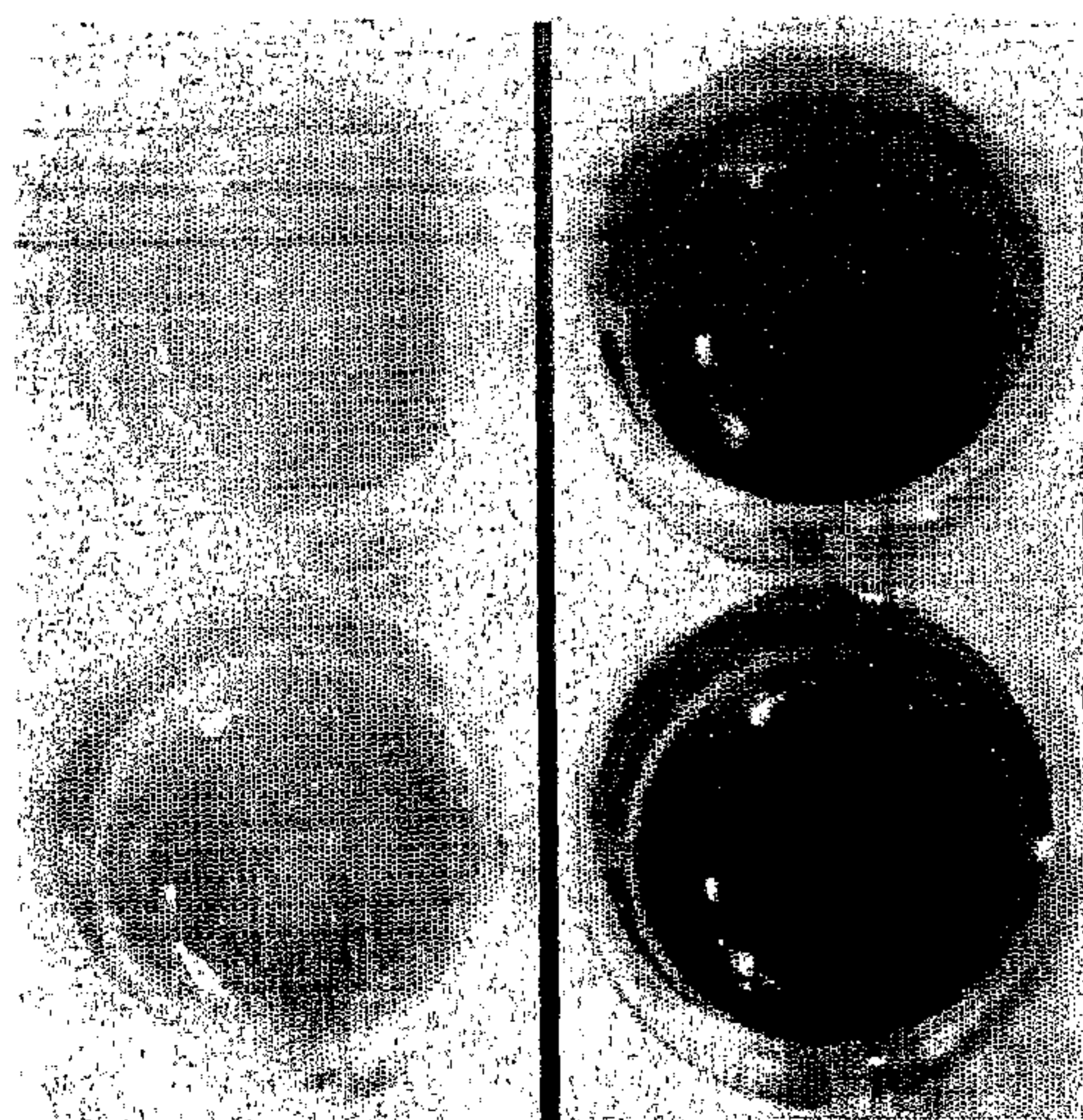
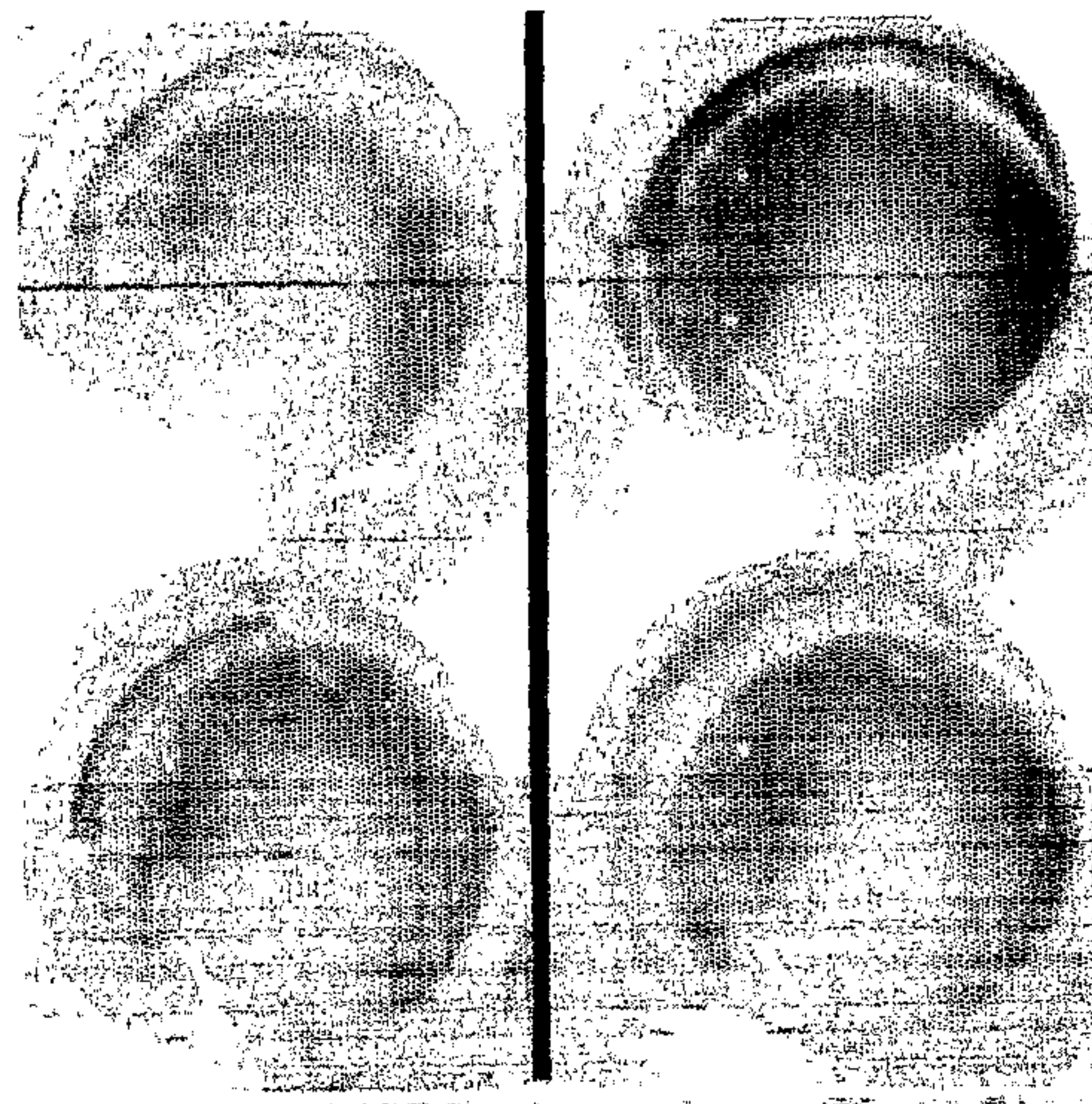
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Figure 9

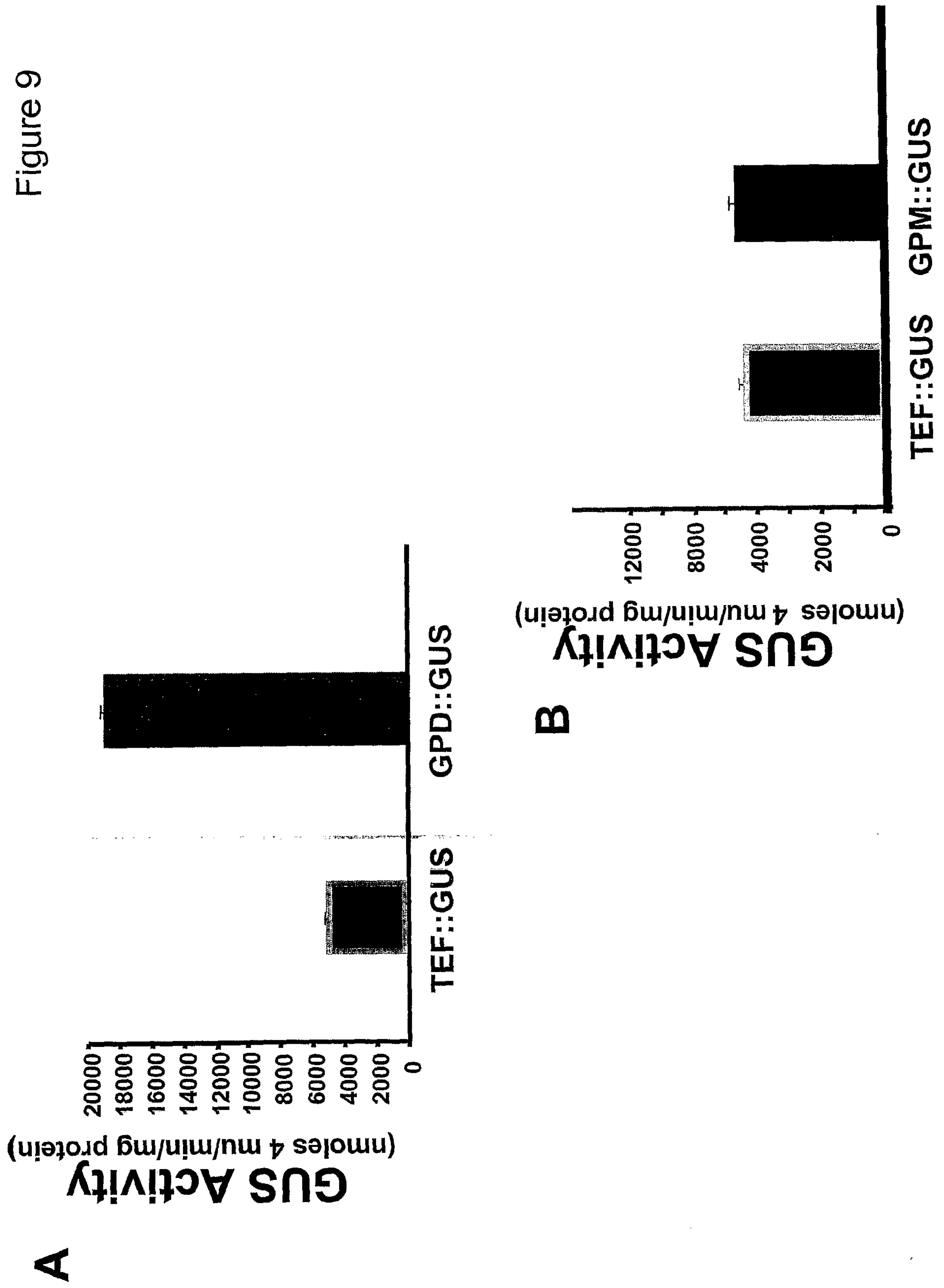


Figure 10

