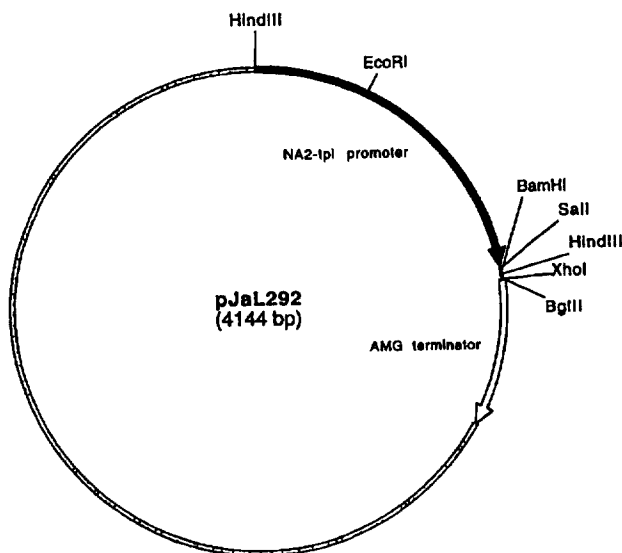




INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification ⁶ : C12N 5/10, C12P 21/00	A1	(11) International Publication Number: WO 98/11203 (43) International Publication Date: 19 March 1998 (19.03.98)
(21) International Application Number: PCT/US97/16604 (22) International Filing Date: 12 September 1997 (12.09.97) (30) Priority Data: 08/713,312 13 September 1996 (13.09.96) US (71) Applicant: NOVO NORDISK BIOTECH, INC. [US/US]; 1445 Drew Avenue, Davis, CA 95616-4880 (US). (72) Inventors: BRODY, Howard; 5136 El Cemente Avenue, Davis, CA 95616 (US). YAVER, Deborah, S.; 2809 Albany Avenue, Davis, CA 95616 (US). LAMSA, Michael, H.; 641 Bluebird Place, Davis, CA 95616 (US). HANSEN, Kim; Bjornsholmvej 30, DK-3500 Værløse (DK). (74) Agents: ZELSON, Steve, T. et al.; Novo Nordisk of North America, Inc., Suite 6400, 405 Lexington Avenue, New York, NY 10174-6401 (US).		(81) Designated States: AL, AU, BB, BG, BR, CA, CN, CU, CZ, EE, GE, HU, IL, IS, JP, KP, KR, LC, LK, LR, LT, LV, MG, MK, MN, MX, NO, NZ, PL, RO, SG, SI, SK, TR, TT, UA, UZ, VN, ARIPO patent (GH, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG). Published <i>With international search report. Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.</i>

(54) Title: CELLS HAVING DNA INSERTION MUTATIONS WHICH PRODUCE ALTERED AMOUNTS OF A POLYPEPTIDE

**(57) Abstract**

The present invention relates to methods for modifying the production of a polypeptide, comprising: (a) introducing a nucleic acid construct into a cell, wherein the cell comprises a DNA sequence encoding a polypeptide, under conditions in which the nucleic acid construct integrates into the genome of the cell at a locus not within the DNA sequence encoding the polypeptide to produce a mutant cell, wherein the integration of the nucleic acid construct modifies the production of the polypeptide by the mutant cell relative to the cell when the mutant cell and the cell are cultured under the same conditions; and (b) identifying the mutant cell with the modified production of the polypeptide.

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CELLS HAVING DNA INSERTION MUTATIONS WHICH PRODUCE ALTERED AMOUNTS OF A POLYPEPTIDE**Background of the Invention****5 Field of the Invention**

This invention relates to methods for modifying the production of a polypeptide by a cell.

Description of the Related Art

- 10 Several methods have been used to modify the production of polypeptides by mutagenizing cells. For example, the production of proteins has been altered by producing mutant cells by classical mutagenesis which involves treating cells with chemical, physical, and biological agents as mutagenic (mutation inducing) agents to increase the frequency of mutational events.
- 15 Production of proteins also has been modified by mutagenesis of a cell with short sections of double-stranded DNA, consisting of more than 2000 base pairs, called transposons which usually code for resistance to one or sometimes several antibiotics. Transposons are able to move or jump within the genome, even between a bacterial chromosome and a plasmid, and they are able to become integrated in a number of different
- 20 sites on the genome. An insertion of a transposon within a structural gene interrupts the normal nucleotide sequence of the gene so that it can no longer deliver the information for the synthesis of the normal, functional polypeptide (Seifert *et al.*, 1986, *Proceedings of the National Academy of Sciences USA* 83: 735-739). An insertion also may disrupt a gene whose gene product is required for expression (Márquez-Magaña and Chamberlin, 1994,
- 25 *Journal of Bacteriology* 176: 2427-2434). In addition, Errede *et al.* (1980, *Cell* 22: 427-436) disclose the insertion of a transposable element adjacent to the structural gene coding for iso-2-cytochrome c causing overproduction. Furthermore, WO 96/29414 discloses that transposable elements may be constructed containing a transposon and a DNA sequence capable of regulating a targeted gene where upon introduction into a cell the transposable
- 30 element integrates into the genome of the cell in a manner which regulates the expression of the gene.

A widely used method for increasing production of a polypeptide is amplification to produce multiple copies of the gene encoding the polypeptide. For example, U.S. Patent No.

5,578,461 discloses the inclusion via homologous recombination of an amplifiable selectable marker gene in tandem with the gene where cells containing amplified copies of the selectable marker can be selected for by culturing the cells in the presence of the appropriate selectable agent.

5 In addition, the production of polypeptides has been increased by replacing one promoter with a different promoter or one signal peptide coding region with another. See, *e.g.*, U.S. Patent No. 5,641,670.

Methods for altering gene expression by disrupting genes encoding various regulatory elements have also been described. For example, Toma *et al.* (1986, *Journal of Bacteriology* 10 167: 740-743) showed that a deletion from -156 to -90 in the *npr* promoter region caused overexpression of the neutral protease encoded by the *npr* gene. Pero and Sloma (1993, *In* A.L. Sonensheim, J.A. Hoch, and R. Losick, editors, *Bacillus subtilis and Other Gram-Positive Bacteria*, pp. 939-952, American Society for Microbiology, Washington, DC) disclose that mutating the sporulation gene *spoOA* results in deficient synthesis of proteases 15 and that mutations in the *abrB* gene restore synthesis.

The production of polypeptides also has been increased by disrupting DNA sequences encoding a protease capable of hydrolyzing the polypeptide under the conditions for producing the polypeptide.

The secretion of polypeptides has also been modified by overproduction of secretion 20 proteins (Ruohonen *et al.*, 1997, *Yeast*, 13: 337-351), and producing a super-secreting cell (U.S. Patent No. 5,312,735).

Methods for increasing the production of metabolites have also been described. For example, WO 96/41886 discloses that increased production of clavam produced by an organism having at least part of the clavam pathway and at least part of a cephalosporin 25 pathway by interfering with the conversion of L-lysine to L-alpha-aminoadipic acid in the cephalosporin pathway. WO 94/13813 discloses the disruption of gene which encodes a protein which degrades betaine, an enzyme inducer.

It is an object of the present invention to provide new and improved methodologies for altering production of polypeptides and metabolites.

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Brief Summary of the Invention

The present invention is drawn to methods for modifying the production of a polypeptide by a cell. In the methods of the present invention, a nucleic acid construct is introduced into a cell which contains a DNA sequence encoding a specific polypeptide. The introduced nucleic acid construct integrates into the host genome at a locus not within the DNA sequence of interest to produce a mutant cell. The integration of the nucleic acid construct into the locus modifies the production of the polypeptide by the mutant cell relative to the parent cell. Mutant cells are then identified in which the polypeptide's production is modified by the mutant cell relative to the parent cell. Modification is determined by comparing production of the polypeptide when the mutant cell and the parent cell are cultured under the same conditions.

An advantage of the present invention is that the mutation can be recovered and leads to a modification of the production of a polypeptide encoded by a DNA sequence which does not contain the mutation.

Brief Description of the Figures

Figure 1 is a restriction map of pJaL292.

Figure 2 is a restriction map of pKS6.

Figure 3 is a restriction map of pBANE13.

Figure 4 is a restriction map of pBANE6.

Figure 5 is a restriction map of pMHan37.

Figure 6 is a restriction map of pBANE8.

Figure 7 is a restriction map of pSO2.

Figure 8 is a restriction map of pSO122 and shows the construction of pDSY81 and pDSY82 from pSO122.

Figure 9 is the nucleic acid sequence and the deduced amino acid sequence of the rescued locus of mutant *Aspergillus oryzae* DEBY599.3 (SEQ ID NOS:9 and 10, respectively).

Figure 10 is the nucleic acid sequence and the deduced amino acid sequence of the rescued locus of mutant *Aspergillus oryzae* DEBY10.3 (SEQ ID NOS:16 and 17).

Figure 11 is a restriction map of pJaL400.

Figure 12 is the construction of pMT1935.

Figure 13 is a restriction map of pJaL394.

Figure 14 is a restriction map of pMT1931.

Figure 15 is a restriction map of pMT1936.

Figure 16 is the nucleic acid sequence and the deduced amino acid sequence of the
5 rescued locus of mutant *Aspergillus oryzae* DEBY932 (SEQ ID NOS:25 and 26).

Figure 17 is the nucleic acid sequence and deduced amino acid sequence of the
rescued locus of mutant *Aspergillus oryzae* DEBY1058 (SEQ ID NOS:29 and 30).

Figure 18 is a restriction map of pDSY161.

Figure 19 is a restriction map of pDSY162.

10 Figure 20 is the nucleic acid sequence of the rescued locus of mutant *Aspergillus*
oryzae 1204.3.3 (SEQ ID NO:34).

Figure 21 is the nucleic acid sequence of the rescued locus of mutant *Aspergillus*
oryzae H603 (SEQ ID NO:39).

Figure 22 is a restriction map of pGAG3.

15 Figure 23 is a restriction map of pJaL389.

Figure 24 is a restriction map of pJaL335.

Figure 25 is a restriction map of pJaL399.

Figure 26 is a restriction map of pDM176.

Figure 27 is a restriction map of pHB218.

20 Figure 28 is a restriction map of pSE39.

Figure 29 is a restriction map of pDSY153.

Figure 30 is a restriction map of pCaHj505.

Figure 31 is a restriction map of pMStr107.

Figure 32 is the nucleic acid sequence and deduced amino acid sequence of the
25 rescued locus of mutant *Aspergillus oryzae* P4-8.1 (SEQ ID NOS:50 and 51).

Figure 33 is the nucleic acid sequence and deduced amino acid sequence of the
rescued locus of mutant *Aspergillus oryzae* P7-14.1 (SEQ ID NOS:56 and 57).

Figure 34 is a restriction map of pMT1612.

Figure 35 is the nucleic acid sequence and deduced amino acid sequence of the
30 rescued locus of mutant *Aspergillus oryzae* DEBY7-17.2 (SEQ ID NOS:63 and 64).

Figure 36 is the nucleic acid sequence of the rescued locus of mutant *Aspergillus*
oryzae DEBY3-2.1 (SEQ ID NO:66).

Figure 37 is the nucleic acid sequence of the rescued locus of mutant *Aspergillus oryzae* DEBY5-7.1 (SEQ ID NO:71).

Figure 38 is the nucleic acid sequence of the rescued locus of mutant *Aspergillus oryzae* DEBY8-10.1 (SEQ ID NO:76).

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Detailed Description of the Invention

In a first embodiment, the present invention relates to methods of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the
10 polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates
15 transcription, translation or secretion of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

20 (b) recovering the polypeptide.

A "protein that negatively regulates transcription" is defined herein as a repressor that negatively affects the process of RNA synthesis by RNA polymerase to produce a single-stranded RNA complementary to a DNA sequence, or as a protein that degrades an enzyme inducer which is generally a chemical agent produced by a biosynthetic or catabolic pathway
25 of a cell. The repressor consists of distinct domains that are required for DNA-binding, transcription repression, and inducer or repressor binding.

A "protein that negatively regulates translation" is defined herein as a protein or a substance, the production of which is catalyzed by the protein, that negatively affects the process of protein synthesis carried out by ribosomes which de-code the information
30 contained in mRNA derived from transcription of a gene. For example, the substance may be a cap-dependent translation initiation factor, *e.g.*, p20 (Altmann *et al.*, 1997, *EMBO*

Journal 16: 1114-1121); or a sex-lethal protein, *e.g.*, the sex-lethal protein of *Drosophila* which regulates the translation of *msl-2* (Bashaw and Baker, 1997, *Cell* 89: 789-798).

A "protein that negatively regulates secretion" is defined herein as a protein or a substance, the production of which is catalyzed by the protein, that negatively affects the process of transferring a protein molecule through a membrane into (i) an intracellular compartment, *e.g.*, a vacuole or mitochondrion, (ii) the periplasmic space, or (iii) the culture medium and, in eukaryotic cells, the process of vesicular transport that ultimately results in exocytic release of secreted proteins from the cell. The secretory process oversees and promotes correct protein folding, mediates any required post-translational modifications (such as glycosylation), and sorts, processes, and targets proteins to specific cellular sites all at a rate consistent with the function of the cell as a whole. Such substances include a protein with Ca^{2+} -ATPase activity which upon inactivation increase levels of secreted heterologous or mutant proteins (for example, see Rudolph *et al.*, 1989, *Cell* 58: 133-145); or the binding protein BiP which is an ATP-dependent hsp70-class chaperone found in the endoplasmic reticulum of eukaryotic cells which when decreased in mammalian cells through the use of anti-sense RNA results in up to a three-fold increase in secreted levels of a mutant protein (Dorner *et al.*, 1988, *Molecular and Cellular Biology* 8: 4063-4070). In a specific embodiment, the substance is a protein with ATPase activity or the binding protein BiP.

In a second embodiment, the present invention relates to methods of producing a polypeptide, comprising

(A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

A mutant cell that "produces" more of a polypeptide is defined herein as a cell from which more of the polypeptide is recovered relative to the parent cell.

In a third embodiment, the present invention relates to methods of producing a polypeptide, comprising

- 5 (a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein
 - (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence,
10 not within a second DNA sequence encoding a protein that negatively regulates transcription of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and
 - (ii) the mutant cell expresses more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and
- 15 (b) recovering the polypeptide.

In a fourth embodiment, the present invention relates to methods of producing a polypeptide, comprising

- (A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein
 - 20 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or
25 regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and
 - (ii) the mutant cell expresses more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and
- (B) recovering the polypeptide.

30 A mutant cell that "expresses" more of a polypeptide is defined herein as a cell that contains an increase in functional mRNA encoding the polypeptide relative to the parent cell. It will be understood that an increase in functional mRNA may result from an increase in the

absolute rate of transcription of the gene encoding the polypeptide and/or from alterations in post-transcriptional processing or modification of the transcripts, including nuclear-cytoplasmic transport and/or cytoplasmic stabilization of the mRNA. Such mutant cells may be identified using conventional techniques, including without limitation Northern blot
5 analysis, run-off transcription assays, and the like.

In a fifth embodiment, the present invention relates to methods of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

10 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates translation of the polypeptide, and not within a third DNA sequence encoding a
15 protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell synthesizes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

In a sixth embodiment, the present invention relates to methods of producing a
20 polypeptide, comprising

(A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into
25 the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

30 (ii) the mutant cell synthesizes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

A mutant cell that "synthesizes" more of a polypeptide is defined herein as a cell that accumulates larger amounts of the polypeptide relative to a parent cell. Accumulation refers to the total amount of the polypeptide in the culture as a whole, *i.e.*, in both intracellular and extracellular compartments taken together. Such mutant cells may be identified using any
5 suitable technique, including without limitation pulse-labelling or steady-state labelling using radiolabelled amino acids; immunoblot analysis of cell and medium fractions using an antibody specific to the polypeptide; assays of biological activity; separation by conventional chromatographic methods; and the like.

In a seventh embodiment, the present invention relates to methods of producing a
10 polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into
15 the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates secretion of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell secretes more of the polypeptide than the parent cell
20 when both cells are cultivated under the conditions;

(b) recovering the polypeptide.

In an eighth embodiment, the present invention relates to methods of producing a polypeptide, comprising

(A) cultivating a mutant cell under conditions conducive for production of the
25 polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an
30 oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell secretes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

A mutant cell that "secretes" more of a polypeptide is defined herein as a cell in which the amount of the polypeptide released into the extracellular medium is increased relative to the parent cell. Such mutant cells may be identified using, *e.g.*, pulse-chase labelling in conjunction with immunoprecipitation to quantify the proportion of the newly synthesized polypeptide that is externalized as well as the absolute amount released in the mutant cell relative to the parent cell. Immunoblot analysis, biological activity assays, and physical-chemical separation methods may also be used to quantify the absolute amounts of the polypeptide released in mutant vs. parent cells.

In a ninth embodiment, the present invention relates to methods of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the random integration of a nucleic acid construct into the genome of the parent cell at a locus wherein the nucleic acid construct is not homologous with the locus and wherein the locus is not within the first DNA sequence nor within a second DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

In a tenth embodiment, the present invention relates to methods of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence

and a second DNA sequence encoding a protein that positively regulates transcription, translation or secretion of the polypeptide; and

(ii) the mutant cell produces less of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

5 (b) recovering the polypeptide.

A mutant cell that "produces" less of a polypeptide is defined herein as a cell from which less of the polypeptide is recovered relative to the parent cell.

A "protein that positively regulates transcription" is defined herein as an activator or an inducer that positively affects the process of RNA synthesis by RNA polymerase to
10 produce a single-stranded RNA complementary to a DNA sequence. The activator consists of distinct domains that are required for DNA-binding, transcription activation, and inducer or repressor binding. An inducer is generally a chemical agent produced by a biosynthetic or catabolic pathway of a cell. In a specific embodiment, the substance is an activator or an inducer.

15 A "protein that positively regulates translation" is defined herein as a protein or a substance, the production of which is catalyzed by the protein, that positively affects the process of protein synthesis carried out by ribosomes which de-code the information contained in mRNA derived from transcription of a gene. In a specific embodiment, the substance is an initiation factor or an elongation factor.

20 A "protein that positively affects secretion" is defined herein as a protein or a substance, the production of which is catalyzed by the protein, that positively affects the process of transferring a protein molecule through a membrane into (i) an intracellular compartment, *e.g.*, a vacuole or mitochondrion, (ii) the periplasmic space, or (iii) the culture medium or positively affects vesicular transport as described above. Such substances
25 include folding proteins, *e.g.*, protein disulfide isomerase and peptidyl prolyl isomerase isoforms; chaperones, *e.g.*, heat shock proteins, signal recognition particles, PrsA, SecD, SecF, and BiP; translocating chain-associating membrane proteins (TRAM); translocase complexes; and processing enzymes, *e.g.*, glycosylating enzymes; signal peptidases; pro region peptidases. In a specific embodiment, the substance is a folding protein, a chaperone,
30 a signal recognition particle, PrsA, SecD, SecF, BiP, a translocating chain-associating membrane, a translocase complex, or a processing enzyme.

Other embodiments of the present invention relate to methods for producing polypeptides as described in the tenth embodiment, except that the mutant cells express, synthesize or secrete less of the polypeptide than the parent cell when both cells are cultivated under the conditions.

5 A mutant cell that "expresses" less of a polypeptide is defined herein as a cell that contains a decrease in functional mRNA encoding the polypeptide relative to the parent cell. It will be understood that a decrease in functional mRNA may result from a decrease in the absolute rate of transcription of the gene encoding the polypeptide and/or from alterations in post-transcriptional processing or modification of the transcripts, including nuclear-
10 cytoplasmic transport and/or cytoplasmic stabilization of the mRNA. Such mutant cells may be identified using conventional techniques, including without limitation Northern blot analysis, run-off transcription assays, and the like.

A mutant cell that "synthesizes" less of a polypeptide is defined herein as a cell that accumulates smaller amounts of the polypeptide relative to a parent cell. Accumulation
15 refers to the total amount of the polypeptide in the culture as a whole, *i.e.*, in both intracellular and extracellular compartments taken together. Such mutant cells may be identified using any suitable technique, including without limitation pulse-labelling or steady-state labelling using radiolabelled amino acids; immunoblot analysis of cell and medium fractions using an antibody specific to the polypeptide; assays of biological activity;
20 separation by conventional chromatographic methods; and the like.

A mutant cell that "secretes" less of a polypeptide is defined herein as a cell in which the amount of the polypeptide released into the extracellular medium is decreased relative to the parent cell. Such mutant cells may be identified using, *e.g.*, pulse-chase labelling in conjunction with immunoprecipitation to quantify the proportion of the newly synthesized
25 polypeptide that is externalized as well as the absolute amount released in the mutant cell relative to the parent cell. Immunoblot analysis, biological activity assays, and physical-chemical separation methods may also be used to quantify the absolute amounts of the polypeptide released in mutant vs. parent cells.

The present invention also relates to methods of producing a metabolite, comprising
30 (A) cultivating a mutant cell under conditions conducive for production of the metabolite, wherein

(i) the mutant cell is related to a parent cell, which comprises one or more first DNA sequences encoding first polypeptides in the biosynthetic pathway of the metabolite, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within (a) the first DNA sequences, (b) a second DNA sequence encoding a substance that negatively regulates transcription, translation or secretion of the polypeptides, (c) a third DNA sequence encoding a protease capable of hydrolyzing any of the first polypeptides under the conditions, and (d) one or more fourth DNA sequences encoding a second polypeptide in the second biosynthetic pathway of a second metabolite wherein the biosynthetic pathway and the second biosynthetic pathway involve the production of the same intermediate and the second polypeptide catalyzes a step after the production of the intermediate; and

(ii) the mutant cell produces more of the metabolite than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the metabolite.

The present invention also relates to methods of producing a metabolite, comprising (A) cultivating a mutant cell under conditions conducive for production of the metabolite, wherein

(i) the mutant cell is related to a parent cell, which comprises one or more first DNA sequences encoding first polypeptides in the biosynthetic pathway of the metabolite, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within (a) the first DNA sequences, (b) a second DNA sequence encoding a protein that negatively regulates transcription, translation or secretion of the polypeptides, and (c) one or more third DNA sequences encoding a second polypeptide in the second biosynthetic pathway of a second metabolite wherein the biosynthetic pathway and the second biosynthetic pathway involve the production of the same intermediate and the second polypeptide catalyzes a step prior to the production of the intermediate; and

(ii) the mutant cell produces less of the metabolite than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the metabolite.

The present invention also relates to methods of producing a first polypeptide, comprising

(a) forming a mutant cell by introducing a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, a second DNA sequence encoding a protein that negatively regulates transcription, translation or secretion of a second polypeptide, and a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under conditions conducive to the production of the first polypeptide;

(b) isolating the mutant cell which produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions;

(c) identifying the locus wherein the nucleic acid construct has been integrated;

10 (d) producing a cell in which a corresponding locus has been disrupted;

(e) culturing the cell under the conditions conducive; and

(f) recovering the first polypeptide.

A corresponding locus is defined herein as a locus which encodes a polypeptide with has the same function as the polypeptide encoded by the rescued locus.

15 The present invention also relates to methods of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

20 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence and a second DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions, wherein the introduction of the nucleic acid construct specifically enhances transcription, translation or secretion of the polypeptide; and

25 (ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

"Specific" enhancement of transcription, translation, or secretion as used herein refers to an enhancement of one or more aspects of the biogenesis and production of the polypeptide that is limited to the polypeptide of interest and is not accompanied by a global effect on other polypeptides in the cell. Preferably, specific enhancement affects only a small

30

number of polypeptides, including the polypeptide of interest. Most preferably, specific enhancement affects only the polypeptide of interest.

Global enhancement of these biogenetic processes can be distinguished from specific enhancement using conventional methods that are well known in the art. For example, biosynthetic pulse-labelling with ^3H -uridine (followed by quantitation of total radioactivity incorporated into RNA) can be used to determine that a mutant cell does not generally synthesize mRNA at a higher or lower rate than the parent cell to which it is related. Similarly, pulse-labelling with, *e.g.*, ^{35}S -methionine (followed by quantitation of total radioactivity incorporated into protein) can be used to determine that a mutant cell does not generally synthesize proteins at a higher or lower rate than the parent cell to which it is relate. General rates of secretion can be compared between mutant and parent cells by pulse-chase labelling using radioactive amino acids or sugars followed by quantitation of extracellular vs. intracellular radioactivity.

These methods can also be used to determine if transcription, translation, or secretion of a limited number of other polypeptides might also be affected in the mutant cell. For example, capture of specific radiolabelled RNA transcripts by hybridization to an immobilized oligonucleotide probe can be used to assess transcription rates of individual genes. For translation and secretion, resolution of radiolabelled nascent proteins by, *e.g.*, SDS-PAGE (with or without immunoprecipitation of individual proteins) can be used to compare instantaneous rates of translation and/or secretion of individual proteins.

Polypeptides

The term "polypeptide" encompasses peptides, oligopeptides, and proteins and, therefore, is not limited to a specific length of the encoded product. The polypeptide may be native to the cell or may be a heterologous polypeptide. Preferably, it is a heterologous polypeptide. The polypeptide may also be a recombinant polypeptide which is a polypeptide native to a cell, which is encoded by a nucleic acid sequence which comprises one or more control sequences foreign to the gene. The polypeptide may be a wild-type polypeptide or a variant thereof. The polypeptide may also be a hybrid polypeptide which contains a combination of partial or complete polypeptide sequences obtained from at least two different polypeptides where one or more of the polypeptides may be heterologous to the cell.

Polypeptides further include naturally occurring allelic and engineered variations of the above mentioned polypeptides.

In a preferred embodiment, the polypeptide is an antibody or portions thereof.

In a preferred embodiment, the polypeptide is an antigen.

5 In a preferred embodiment, the polypeptide is a clotting factor.

In a preferred embodiment, the polypeptide is an enzyme.

In a preferred embodiment, the polypeptide is a hormone or a hormone variant.

In a preferred embodiment, the polypeptide is a receptor or portions thereof.

In a preferred embodiment, the polypeptide is a regulatory protein.

10 In a preferred embodiment, the polypeptide is a structural protein.

In a preferred embodiment, the polypeptide is a reporter.

In a preferred embodiment, the polypeptide is a transport protein.

In a more preferred embodiment, the polypeptide is an oxidoreductase,

In a more preferred embodiment, the polypeptide is a transferase.

15 In a more preferred embodiment, the polypeptide is a hydrolase.

In a more preferred embodiment, the polypeptide is a lyase.

In a more preferred embodiment, the polypeptide is an isomerase.

In a more preferred embodiment, the polypeptide is a ligase.

In an even more preferred embodiment, the polypeptide is an aminopeptidase

20 In an even more preferred embodiment, the polypeptide is an amylase.

In an even more preferred embodiment, the polypeptide is a carbohydrase.

In an even more preferred embodiment, the polypeptide is a carboxypeptidase.

In an even more preferred embodiment, the polypeptide is a catalase.

In an even more preferred embodiment, the polypeptide is a cellulase.

25 In an even more preferred embodiment, the polypeptide is a chitinase.

In an even more preferred embodiment, the polypeptide is a cutinase.

In an even more preferred embodiment, the polypeptide is a deoxyribonuclease.

In an even more preferred embodiment, the polypeptide is a dextranase.

In an even more preferred embodiment, the polypeptide is an esterase.

30 In an even more preferred embodiment, the polypeptide is an alpha-galactosidase.

In an even more preferred embodiment, the polypeptide is a beta-galactosidase.

In an even more preferred embodiment, the polypeptide is a glucoamylase.

- In an even more preferred embodiment, the polypeptide is an alpha-glucosidase.
- In an even more preferred embodiment, the polypeptide is a beta-glucosidase.
- In an even more preferred embodiment, the polypeptide is a haloperoxidase.
- In an even more preferred embodiment, the polypeptide is an invertase.
- 5 In an even more preferred embodiment, the polypeptide is a laccase.
- In an even more preferred embodiment, the polypeptide is a lipase.
- In an even more preferred embodiment, the polypeptide is a mannosidase.
- In an even more preferred embodiment, the polypeptide is a mutanase.
- In an even more preferred embodiment, the polypeptide is an oxidase.
- 10 In an even more preferred embodiment, the polypeptide is a pectinolytic enzyme.
- In an even more preferred embodiment, the polypeptide is a peroxidase.
- In an even more preferred embodiment, the polypeptide is a phytase.
- In an even more preferred embodiment, the polypeptide is a polyphenoloxidase.
- In an even more preferred embodiment, the polypeptide is a proteolytic enzyme.
- 15 In an even more preferred embodiment, the polypeptide is a ribonuclease.
- In an even more preferred embodiment, the polypeptide is a transglutaminase.
- In an even more preferred embodiment, the polypeptide is a xylanase.
- In an even more preferred embodiment, the polypeptide is human insulin or an analog thereof.
- 20 In an even more preferred embodiment, the polypeptide is human growth hormone.
- In an even more preferred embodiment, the polypeptide is erythropoietin.
- In an even more preferred embodiment, the polypeptide is insulinotropin.

The polypeptide also may be an enzyme involved in the biosynthesis of a specific metabolite. The biosynthesis of a metabolite generally involves a biosynthetic pathway

25 containing an array of enzyme-catalyzed chemical reaction steps in which one or more steps may be rate-limiting. In this embodiment of the present invention, the integration of the nucleic acid construct into the cell's genome modifies the production of the metabolite by modifying one or more of these enzyme-catalyzed steps.

The metabolite may be any organic compound of a cell which has been produced by

30 transformation of a precursor organic compound by an enzyme-catalyzed chemical reaction of the cell. The metabolite may be a primary metabolite or a secondary metabolite. Furthermore, the metabolite may be a biosynthetic pathway intermediate or a biosynthetic

pathway product. Preferably, the metabolite is an alkaloid, an amino acid, an antibiotic, a cofactor, a drug, a fatty acid, a fungicide, a herbicide, an insecticide, an organic acid, a prosthetic group, a rodenticide, a sweetener, a vitamin, a deoxysugar, a surfactant, a mycotoxin, an organic acid, a sugar alcohol, a toxic metabolite, or a toxin.

5

Nucleic Acid Constructs

The nucleic constructs used in the methods of the present invention may be termed "tagged nucleic acid constructs". "A tagged nucleic acid construct" is a nucleic acid molecule containing an identifiable nucleic acid sequence which integrates into the cell's
10 genome at one or more loci thereby marking the loci. The genome is the complete set of DNA of a cell including chromosomal and artificial chromosomal DNA and extrachromosomal DNA, *i.e.*, self-replicative genetic elements.

The nucleic acid constructs may be any nucleic acid molecule, either single- or double-stranded, which is synthetic DNA, isolated from a naturally occurring gene, or has
15 been modified to contain segments of nucleic acid which are combined and juxtaposed in a manner which would not otherwise exist in nature. The nucleic acid constructs may be circular or linear. Furthermore, the nucleic acid constructs may be contained in a vector, may be a restriction enzyme cleaved linearized fragment, or may be a PCR amplified linear fragment.

20 The nucleic acid constructs may contain any nucleic acid sequence of any size. In one embodiment, the nucleic acid constructs are between about 10 - 20,000 bp in length, preferably 100 - 15,000 bp in length, more preferably 500 - 15,000 bp in length, even more preferably 1000 - 15,000 bp in length, and most preferably 1,000 - 10,000 bp in length.

25 Preferably, the nucleic acid constructs have less than 40% homology, preferably less than 30% homology, more preferably less than 20% homology, even more preferably less than 10% homology, and most preferably no homology with the locus.

30 Preferably, the nucleic acid constructs have less than 40% homology, preferably less than 30% identity, more preferably less than 20% identity, even more preferably less than 10%, and most preferably no homology with the DNA sequence encoding the polypeptide of interest.

The nucleic acid construct can be introduced into a cell as two or more separate fragments. In the event two fragments are used, the two fragments share DNA sequence

homology (overlap) at the 3' end of one fragment and the 5' end of the other. Upon introduction into a cell, the two fragments can undergo homologous recombination to form a single fragment. The product fragment is then in a form suitable for recombination with the cellular sequences. More than two fragments can be used, designed such that they will
5 undergo homologous recombination with each other to ultimately form a product suitable for recombination with a cellular sequence.

It will be further understood that two or more nucleic acid constructs may be introduced into the cell as circular or linear fragments using the methods of the present invention, wherein the fragments do not contain overlapping regions as described above. It
10 is well known in the art that for some organisms, the introduction of multiple constructs into a cell results in their integration at the same locus.

The nucleic acid constructs can contain coding or non-coding DNA sequences. Coding sequences are sequences which are capable of being transcribed into mRNA and translated into a polypeptide when placed under the control of the appropriate control
15 sequences. The boundaries of a coding sequence are generally determined by a translation start codon ATG at the 5'-terminus and a translation stop codon at the 3'-terminus. A coding sequence can include, but is not limited to, DNA, cDNA, and recombinant nucleic acid sequences.

In a preferred embodiment, the nucleic acid constructs contain a selectable marker as
20 the identifiable nucleic acid sequence. A selectable marker is a gene, the product of which provides for biocide or viral resistance, resistance to heavy metals, prototrophy to auxotrophs, and the like. Integration of a selectable marker into the genome of a host cell permits easy selection of transformed cells. Selectable marker genes for use in the methods of the present invention include, but are not limited to, acetamidase (*amdS*), 5-aminolevulinic
25 acid synthase (*hemA*), anthranilate synthase (*trpC*), glufosinate resistance genes, hygromycin phosphotransferase (*hygB*), nitrate reductase (*niaD*), ornithine carbamoyltransferase (*argB*), orotidine-5'-phosphate decarboxylase (*pyrG*), phosphinothricin acetyltransferase (*bar*), and sulfate adenylyltransferase (*sC*), as well as equivalents from other species. In a more preferred embodiment, the selectable marker is the *amdS* gene of *Aspergillus nidulans* or *Aspergillus*
30 *oryzae*, the *bar* gene of *Streptomyces hygroscopicus*, the *hemA* gene of *Aspergillus oryzae* or the *pyrG* gene of *Aspergillus nidulans* or *Aspergillus oryzae*. Other selectable markers for use in the methods of the present invention are the *dal* genes from *Bacillus subtilis* or

Bacillus licheniformis, or markers which confer antibiotic resistance such as ampicillin (*amp*), kanamycin (*kan*), chloramphenicol (*cam*) or tetracycline resistance (*tet*). A frequently used mammalian marker is the dihydrofolate reductase gene (*dhfr*). Suitable markers for yeast host cells are ADE2, HIS3, LEU2, LYS2, MET3, TRP1, and URA3.

5 In another preferred embodiment, the constructs comprise vector sequences alone or in combination with a selectable marker, including vector sequences containing an origin of replication, *e.g.*, *E. coli* vector sequences such as pUC19, pBR322, or pBluescript. For example, an *E. coli* vector sequence containing an origin of replication can facilitate recovery of the construct from the host genome after integration due to the *E. coli* origin of
10 replication. The construct can be recovered from the host genome by digestion of the genomic DNA with a restriction endonuclease followed by ligation of the recovered construct and transformation of *E. coli*.

In a preferred embodiment, the nucleic acid constructs do not contain the coding sequence of the DNA sequence for the polypeptide or portions thereof. In another preferred
15 embodiment, the nucleic acid constructs contain a sequence which is not homologous to the DNA sequence encoding the polypeptide in order to block the construct from integrating or disrupting the DNA sequence of interest.

In another preferred embodiment, the nucleic acid constructs contain one or more copies of the DNA sequence coding for the polypeptide operably linked to control sequences.
20 In this embodiment, the production of the polypeptide will be modified by both gene inactivation and the introduction of one or more copies of the DNA sequence.

In another preferred embodiment, the nucleic acid constructs do not contain transposable elements, *i.e.*, transposons. A transposon is a discrete piece of DNA which can insert itself into many different sites in other DNA sequences within the same cell. The
25 proteins necessary for the transposition process are encoded within the transposon. A copy of the transposon may be retained at the original site after transposition. The ends of a transposon are usually identical but in inverse orientation with respect to one another.

In another preferred embodiment, the nucleic acid constructs may contain one or more control sequences, *e.g.*, a promoter alone or in combination with a selectable marker,
30 wherein the control sequences upon integration are not operably linked to the DNA sequence encoding the polypeptide of interest. The term "operably linked" is defined herein as a configuration in which a control sequence is appropriately placed at a position relative to the

coding sequence of the DNA sequence such that the control sequence directs the production of a primary RNA transcript. Such control sequences are a promoter, a signal sequence, a propeptide sequence, a transcription terminator, a polyadenylation sequence, an enhancer sequence, an attenuator sequence, and an intron splice site sequence. Each control sequence
5 may be native or foreign to the cell or to the polypeptide-coding sequence.

The presence of a strong promoter in the nucleic acid construct allows for additional genetic effects in addition to gene inactivation via insertion into a structural gene (or functional transcriptional promoter or mRNA termination regions). The promoter may insert upstream of a structural gene so as to enhance its transcription. Alternatively, if the
10 promoter sequences insert in reverse gene orientation so as to generate antisense RNA, there is the possibility of gene inactivation in diploid or higher ploidy cells. By the same mechanism, insertion of the promoter sequences in reverse orientation may result in inactivation of multiple gene family encoded gene product activities.

In another preferred embodiment, the nucleic acid constructs contain a control
15 sequence other than a promoter.

In another preferred embodiment, the nucleic acid constructs do not contain control sequences.

Locus

20 In the methods of the present invention, the nucleic acid constructs are introduced at a "locus not within the DNA sequence of interest" or a "locus not within DNA sequences encoding polypeptides in the biosynthetic pathway of a metabolite" which means that the nucleic acid construct is not introduced into the polypeptide-coding sequence, the control sequences thereof, and any intron sequences within the coding sequence.

25 Control sequences include all components which are operably linked to the DNA sequence and involved in the expression of the polypeptide-coding sequence. Such control sequences are a promoter, a signal sequence, a propeptide sequence, a transcription terminator, a polyadenylation sequence, an enhancer sequence, an attenuator sequence, and an intron splice site sequence. Each of the control sequences may be native or foreign to the
30 coding sequence.

The promoter sequence contains transcriptional control sequences which mediate the expression of the polypeptide. The promoter may be any promoter sequence including mutant, truncated, and hybrid promoters.

The signal peptide coding region codes for an amino acid sequence linked to the amino terminus of the polypeptide which can direct the expressed polypeptide into the cell's secretory pathway.

The propeptide coding region codes for an amino acid sequence positioned at the amino terminus of the polypeptide. The resultant polypeptide is known as a proenzyme or propolypeptide (or a zymogen in some cases). A propolypeptide is generally inactive and can be converted to mature active polypeptide by catalytic or autocatalytic cleavage of the propeptide from the propolypeptide.

The terminator is a sequence operably linked to the 3' terminus of the polypeptide coding sequence, and is recognized by the cell to terminate transcription of the polypeptide coding sequence.

The polyadenylation sequence is a sequence which is operably linked to the 3' terminus of the DNA sequence and which, when transcribed, is recognized by the cell as a signal to add polyadenosine residues to the transcribed mRNA.

The enhancer sequence is a sequence which can increase transcription from a gene when located up to several kilobases from the gene. The enhancer sequencer is usually upstream of the gene.

The attenuator sequence is a sequence which regulates the expression of a gene by determining whether the mRNA molecule containing its transcript will be completed or not.

The intron sequence is a sequence of a gene which is not represented in the protein product of the gene. Intron sequences are transcribed into RNA and must be excised and the RNA molecule religated through a process called intron splicing before it can be translated.

The locus may be noncontiguous or contiguous with the above-noted sequences. Preferably the locus is noncontiguous. The locus may be on the same chromosome or the same extrachromosomal element or on a different chromosome or a different extrachromosomal element as that of the DNA sequence of interest. Furthermore, the locus may be native or foreign to the cell.

In a preferred embodiment, the locus is at least 1,000 bp, more preferably at least 2,000 bp, and even more preferably at least 3,000 bp, even more preferably at least 4,000

bp, even more preferably at least 5,000 bp, and most preferably at least 10,000 bp from the 5' or 3' terminus of the DNA sequence of interest.

In another preferred embodiment, the locus is on a different chromosome than the DNA sequence encoding the polypeptide of interest.

5 In various methods of the present invention, the nucleic acid constructs are introduced at a locus not within a DNA sequence encoding a protease capable of hydrolyzing the polypeptide under physiological conditions, which means that the nucleic acid construct is not introduced into the protease-coding sequence, the control sequences thereof, any intron sequences within the coding sequence, and any DNA sequences encoding proteins that
10 positively regulate transcription, translation or secretion of the protease.

In another preferred embodiment, the locus encodes a polypeptide different from the polypeptide encoded by the DNA sequence.

In another preferred embodiment, the locus encodes a glucose transporter. Preferably, the locus has at least 60% homology, more preferably at least 70% homology,
15 even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:9.

In another preferred embodiment, the locus encodes a mannitol-1-phosphate dehydrogenase. Preferably, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least
20 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:25.

In another preferred embodiment, the locus encodes a chitin synthase. Preferably, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most
25 preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:56.

In another preferred embodiment, the locus encodes a heat shock protein. Preferably, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:50.

30 In another preferred embodiment, the locus encodes a manganese superoxide dismutase. Preferably, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90%

homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:29.

In another preferred embodiment, the locus is a gene required for activation of *pacC*, preferably a *palB* gene. Preferably, the locus has at least 60% homology, more preferably
5 at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:16.

In another preferred embodiment, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more
10 preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:34.

In another preferred embodiment, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the
15 nucleic acid sequence of SEQ ID NO:39.

In another preferred embodiment, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:63.

20 In another preferred embodiment, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:66.

In another preferred embodiment, the locus has at least 60% homology, more
25 preferably at least 70% homology, even more preferably at least 80% homology, even more preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:71.

In another preferred embodiment, the locus has at least 60% homology, more preferably at least 70% homology, even more preferably at least 80% homology, even more
30 preferably at least 90% homology, and most preferably at least 95% homology with the nucleic acid sequence of SEQ ID NO:76.

In another preferred embodiment, the locus encodes an aminopeptidase.

- In another preferred embodiment, the locus encodes an amylase.
- In another preferred embodiment, the locus encodes a carbohydrase.
- In another preferred embodiment, the locus encodes a carboxypeptidase.
- In another preferred embodiment, the locus encodes a catalase.
- 5 In another preferred embodiment, the locus encodes a catalase.
- In another preferred embodiment, the locus encodes a cellulase.
- In another preferred embodiment, the locus encodes a chitinase.
- In another preferred embodiment, the locus encodes a cutinase.
- In another preferred embodiment, the locus encodes a deoxyribonuclease.
- 10 In another preferred embodiment, the locus encodes a dextranase.
- In another preferred embodiment, the locus encodes an esterase.
- In another preferred embodiment, the locus encodes an alpha-galactosidase.
- In another preferred embodiment, the locus encodes a beta-galactosidase.
- In another preferred embodiment, the locus encodes a glucoamylase.
- 15 In another preferred embodiment, the locus encodes an alpha-glucosidase.
- In another preferred embodiment, the locus encodes a beta-galactosidase.
- In another preferred embodiment, the locus encodes a glucoamylase.
- In another preferred embodiment, the locus encodes an alpha-glucosidase.
- In another preferred embodiment, the locus encodes a beta-glucosidase.
- 20 In another preferred embodiment, the locus encodes a haloperoxidase.
- In another preferred embodiment, the locus encodes an invertase.
- In another preferred embodiment, the locus encodes a laccase.
- In another preferred embodiment, the locus encodes a lipase.
- In another preferred embodiment, the locus encodes a mannosidase.
- 25 In another preferred embodiment, the locus encodes a mutanase.
- In another preferred embodiment, the locus encodes an oxidase.
- In another preferred embodiment, the locus encodes a pectinolytic enzyme.
- In another preferred embodiment, the locus encodes a peroxidase.
- In another preferred embodiment, the locus encodes a phytase.
- 30 In another preferred embodiment, the locus encodes a polyphenoloxidase.
- In another preferred embodiment, the locus encodes a proteolytic enzyme.
- In another preferred embodiment, the locus encodes a ribonuclease.

In another preferred embodiment, the locus encodes a transglutaminase.

In another preferred embodiment, the locus encodes a xylanase.

In a more preferred embodiment, the locus is the sequence contained in pDSY109.

In a more preferred embodiment, the locus is the sequence contained in pDSY112.

5 In a more preferred embodiment, the locus is the sequence contained in pDSY138.

In a more preferred embodiment, the locus is the sequence contained in pDSY141.

In a more preferred embodiment, the locus is the sequence contained in pDSY162.

In a more preferred embodiment, the locus is the sequence contained in pMT1936.

In a more preferred embodiment, the locus is the sequence contained in pSMO1204.

10 In a more preferred embodiment, the locus is the sequence contained in pSMOH603.

In a more preferred embodiment, the locus is the sequence of SEQ ID NO:9.

In a more preferred embodiment, the locus is the sequence of SEQ ID NO:16.

In a more preferred embodiment, the locus is the sequence of SEQ ID NO:25.

In a more preferred embodiment, the locus is the sequence of SEQ ID NO:29.

15 In a more preferred embodiment, the locus is the sequence of SEQ ID NO:34.

In a more preferred embodiment, the locus is the sequence of SEQ ID NO:39.

In another more preferred embodiment, the locus is the sequence contained in p4-8.1.

In another more preferred embodiment, the locus is the sequence contained in p7-14.1.

20 In another more preferred embodiment, the locus is the sequence contained in pHB220.

In another more preferred embodiment, the locus is the sequence contained in pSMO717.

25 In another more preferred embodiment, the locus is the sequence contained in pSMO321.

In another more preferred embodiment, the locus is the sequence contained in pHowB571.

In another more preferred embodiment, the locus is the sequence contained in pSMO810.

30 In another more preferred embodiment, the locus is the sequence of SEQ ID NO:50.

In another more preferred embodiment, the locus is the sequence of SEQ ID NO:56.

In another more preferred embodiment, the locus is the sequence of SEQ ID NO:63.

In another more preferred embodiment, the locus is the sequence of SEQ ID NO:66.

In another more preferred embodiment, the locus is the sequence of SEQ ID NO:71.

In another more preferred embodiment, the locus is the sequence of SEQ ID NO:76.

In another preferred embodiment, the locus does not encode a *trans* factor of the DNA sequence of interest. A “*trans* factor” is a factor which is encoded by a gene separate from the DNA sequence of interest which activates or represses transcription of the DNA sequence. In a more preferred embodiment, the locus does not encode a repressor of the DNA sequence of interest. In a more preferred embodiment, the locus does not encode an activator of the DNA sequence of interest.

10

Cells

The methods of the present invention may be used with any cell containing a DNA sequence encoding a polypeptide of interest including prokaryotic cells such as bacteria, or eukaryotic cells such as mammalian, insect, plant, and fungal cells. The DNA sequence may be native or foreign to the cell. The cell may be a unicellular microorganism or a non-unicellular microorganism. Furthermore, the cell may be wild-type or a mutant cell. For example, the mutant cell may be a cell which has undergone classical mutagenesis or genetic manipulation.

Useful prokaryotic cells are bacterial cells such as gram positive bacteria including, but not limited to, a *Bacillus* cell, *e.g.*, *Bacillus alkalophilus*, *Bacillus amyloliquefaciens*, *Bacillus brevis*, *Bacillus circulans*, *Bacillus coagulans*, *Bacillus lautus*, *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus megaterium*, *Bacillus stearothermophilus*, *Bacillus subtilis*, and *Bacillus thuringiensis*; or a *Streptomyces* cell, *e.g.*, *Streptomyces lividans* or *Streptomyces murinus*, or gram negative bacteria such as *E. coli* and *Pseudomonas* sp. In a preferred embodiment, the bacterial cell is a *Bacillus lentus*, *Bacillus licheniformis*, *Bacillus stearothermophilus*, or *Bacillus subtilis* cell.

In a preferred embodiment, the cell is a fungal cell. “Fungi” as used herein includes the phyla Ascomycota, Basidiomycota, Chytridiomycota, and Zygomycota (as defined by Hawksworth *et al.*, In, Ainsworth and Bisby’s Dictionary of The Fungi, 8th edition, 1995, CAB International, University Press, Cambridge, UK) as well as the Oomycota (as cited in Hawksworth *et al.*, 1995, *supra*, page 171) and all mitosporic fungi (Hawksworth *et al.*, 1995, *supra*). Representative groups of Ascomycota include, *e.g.*, *Neurospora*,

Eupenicillium (= *Penicillium*), *Emericella* (= *Aspergillus*), *Eurotium* (= *Aspergillus*), and the true yeasts. Examples of Basidiomycota include mushrooms, rusts, and smuts. Representative groups of Chytridiomycota include, e.g., *Allomyces*, *Blastocladiella*, *Coelomomyces*, and aquatic fungi. Representative groups of Oomycota include, e.g.,
5 Saprolegniomycetous aquatic fungi (water molds) such as *Achlya*. Examples of mitosporic fungi include *Alternaria*, *Aspergillus*, *Candida*, and *Penicillium*. Representative groups of Zygomycota include, e.g., *Mucor* and *Rhizopus*.

In a preferred embodiment, the fungal cell is a yeast cell. "Yeast" as used herein includes ascosporogenous yeast (Endomycetales), basidiosporogenous yeast, and yeast
10 belonging to the Fungi Imperfecti (Blastomycetes). The ascosporogenous yeasts are divided into the families Spermophthoraceae and Saccharomycetaceae. The latter is comprised of four subfamilies, Schizosaccharomycoideae (e.g., genus *Schizosaccharomyces*), Nadsonioideae, Lipomycoideae, and Saccharomycoideae (e.g., genera *Kluyveromyces*, *Pichia*, and *Saccharomyces*). The basidiosporogenous yeasts include the genera *Filobasidiella*,
15 *Filobasidium*, *Leucosporidium*, *Rhodosporidium*, and *Sporidiobolus*. Yeast belonging to the Fungi Imperfecti are divided into two families, Sporobolomycetaceae (e.g., genera *Bullera* and *Sorobolomyces*) and Cryptococcaceae (e.g., genus *Candida*). Since the classification of yeast may change in the future, for the purposes of this invention, yeast shall be defined as described in Biology and Activities of Yeast (Skinner *et al.*, 1980, Soc. App. Bacteriol.
20 Symposium Series No. 9, 1980. The biology of yeast and manipulation of yeast genetics are well known in the art (see, e.g., Biochemistry and Genetics of Yeast, (Bacil, M., Horecker, B.J., and Stopani, A.O.M., editors), 2nd edition, 1987; The Yeasts (Rose, A.H., and Harrison, J.S., editors), 2nd edition, 1987; and The Molecular Biology of the Yeast *Saccharomyces*, Strathern *et al.*, editors, 1981).

25 In a more preferred embodiment, the yeast cell is a cell of a species of *Candida*, *Kluyveromyces*, *Pichia*, *Saccharomyces*, *Schizosaccharomyces*, or *Yarrowia*.

In a most preferred embodiment, the yeast cell is a *Saccharomyces carlsbergensis*, *Saccharomyces cerevisiae*, *Saccharomyces diastaticus*, *Saccharomyces douglasii*, *Saccharomyces kluyveri*, *Saccharomyces norbensis*, or *Saccharomyces oviformis* cell. In
30 another most preferred embodiment, the yeast cell is a *Kluyveromyces lactis* cell. In another most preferred embodiment, the yeast cell is a *Yarrowia lipolytica* cell.

In another preferred embodiment, the fungal cell is a filamentous fungal cell. "Filamentous fungi" include all filamentous forms of the subdivision Eumycota and Oomycota (as defined by Hawksworth *et al.*, 1995, *supra*). The filamentous fungi are characterized by a mycelial wall composed of chitin, cellulose, glucan, chitosan, mannan, and other complex polysaccharides. Vegetative growth is by hyphal elongation and carbon catabolism is obligately aerobic. In contrast, vegetative growth by yeasts such as *Saccharomyces cerevisiae* is by budding of a unicellular thallus and carbon catabolism may be fermentative. In a more preferred embodiment, the filamentous fungal cell is a cell of a species of, but not limited to, *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*, *Neurospora*, *Penicillium*, *Scytalidium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.

In an even more preferred embodiment, the filamentous fungal cell is an *Aspergillus* cell. In another even more preferred embodiment, the filamentous fungal cell is an *Acremonium* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Fusarium* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Humicola* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Mucor* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Myceliophthora* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Neurospora* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Penicillium* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Thielavia* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Tolypocladium* cell. In another even more preferred embodiment, the filamentous fungal cell is a *Trichoderma* cell.

In a most preferred embodiment, the filamentous fungal cell is an *Aspergillus* *awamori*, *Aspergillus foetidus*, *Aspergillus japonicus*, *Aspergillus nidulans*, *Aspergillus niger*, or *Aspergillus oryzae* cell. In another most preferred embodiment, the filamentous fungal cell is a *Fusarium bactridioides*, *Fusarium cerealis*, *Fusarium crookwellense*, *Fusarium culmorum*, *Fusarium graminearum*, *Fusarium graminum*, *Fusarium heterosporum*, *Fusarium negundi*, *Fusarium oxysporum*, *Fusarium reticulatum*, *Fusarium roseum*, *Fusarium sambucinum*, *Fusarium sarcochroum*, *Fusarium sulphureum*, *Fusarium toruloseum*, *Fusarium trichothecioides*, or *Fusarium venenatum* cell. In a most preferred embodiment, the filamentous fungal cell is a *Fusarium venenatum* cell (Nirenberg sp. nov.). In another most

preferred embodiment, the filamentous fungal cell is a *Humicola insolens* cell or a *Humicola lanuginosa* cell. In another most preferred embodiment, the filamentous fungal cell is a *Mucor miehei* cell. In another most preferred embodiment, the filamentous fungal cell is a *Myceliophthora thermophila* cell. In another most preferred embodiment, the filamentous fungal cell is a *Neurospora crassa* cell. In another most preferred embodiment, the filamentous fungal cell is a *Penicillium purpurogenum* cell. In another most preferred embodiment, the filamentous fungal cell is a *Thielavia terrestris* cell. In another most preferred embodiment, the filamentous fungal cell is a *Trichoderma harzianum*, *Trichoderma koningii*, *Trichoderma longibrachiatum*, *Trichoderma reesei*, or *Trichoderma viride* cell.

Useful mammalian cells include Chinese hamster ovary (CHO) cells, HeLa cells, baby hamster kidney (BHK) cells, COS cells, or any number of immortalized cells available, *e.g.*, from the American Type Culture Collection.

Introduction of Nucleic Acid Constructs into Cells

The nucleic acid construct(s) may be introduced into a cell by a variety of physical or chemical methods known in the art including, but not limited to, transfection or transduction, electroporation, microinjection, microprojectile bombardment, alkali salts, or protoplast-mediated transformation.

The introduction of the nucleic acid construct into a cell for insertional mutagenesis is referred to as "DNA-tagged mutagenesis". "DNA-tagged mutagenesis" is defined herein as the introduction of a nucleic acid molecule into a cell, which leads to one or more insertions of the nucleic acid molecule into one or more loci of the genome of the cell thereby marking the loci into which the nucleic acid molecule is inserted. The mutant cell produced by DNA-tagged mutagenesis is called a tagged mutant.

Suitable procedures for transformation of *Aspergillus* cells are described in EP 238 023 and Yelton *et al.*, 1984, *Proceedings of the National Academy of Sciences USA* 81: 1470-1474. A suitable method of transforming *Fusarium* species is described by Malardier *et al.*, 1989, *Gene* 78: 147-156 or in WO 96/00787. Yeast may be transformed using the procedures described by Becker and Guarente, In Guide to Yeast Genetics and Molecular Biology, *Methods of Enzymology* 194: 182-187; Ito *et al.*, 1983, *Journal of Bacteriology* 153: 163; and Hinnen *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75: 1920.

The transformation of a bacterial cell may, for instance, be accomplished by protoplast transformation (see, *e.g.*, Chang and Cohen, 1979, *Molecular General Genetics* 168: 111-115), by using competent cells (see, *e.g.*, Young and Spizizin, 1961, *Journal of Bacteriology* 81: 823-829, or Dubnau and Davidoff-Abelson, 1971, *Journal of Molecular Biology* 56: 209-221), by electroporation (see, *e.g.*, Shigekawa and Dower, 1988, *Biotechniques* 6: 742-751), or by conjugation (see, *e.g.*, Koehler and Thorne, 1987, *Journal of Bacteriology* 169: 5771-5278).

Mammalian cells may be transformed by direct uptake using the calcium phosphate precipitation method of Graham and Van der Eb, 1978, *Virology* 52: 546. Other processes, *e.g.*, electroporation, known to the art, may be used.

When the nucleic acid construct is a vector, integration into the cell's genome occurs randomly by homologous and/or non-homologous recombination depending on the cell of choice.

In a preferred embodiment, the nucleic acid construct is introduced into the parent cell by restriction enzyme-mediated integration (REMI). REMI, which is described in Schiestl and Petes, 1991, *Proceedings of the National Academy of Sciences USA* 88: 7585-7589, is the introduction of plasmid DNA digested with a restriction enzyme along with the restriction enzyme into a cell which subsequently leads to integration of the plasmid DNA into the genome often at a site specified by the restriction enzyme added. The advantage of REMI DNA-tagged mutagenesis is it can generate mutations whose molecular basis can be easily identified.

When the nucleic acid construct is a restriction enzyme cleaved linear DNA fragment, insertion of the construct into the cell's genome through REMI in the presence of the appropriate restriction enzyme is random by virtue of the randomness of the restriction sites present in the genome. The nucleic acid construct may insert into the cell's genome as a single copy or as multiple copies at a single locus or at a different locus or at different loci. It is preferable that the nucleic acid construct insert as a single copy to facilitate the identification and recovery of the tagged locus.

Screening of Mutant Cells

The present invention also relates to mutant cells which produce, express, synthesize or secrete more of a polypeptide or metabolite than the parent cell when both cells are cultivated under the conditions.

5 The present invention also relates to mutant cells which produce, express, synthesize or secrete more of a polypeptide or metabolite than the parent cell when both cells are cultivated under the conditions.

Following the introduction of a nucleic acid construct into a cell, the next step is to isolate the mutant cell with the modified production of a polypeptide from a population of
10 presumptive mutant cells. The isolation of the mutant cell preferably relies on measurement of the production of the polypeptide or the metabolite by the mutant cell relative to the parent cell when the mutant cell and the parent cell are cultured under the same conditions.

The phrase "modified production of a polypeptide" includes an alteration or change of a step in the production of a polypeptide or a metabolite by the mutant cell relative to the
15 parent cell. Such steps include, but are not limited to, transcription, post-transcriptional modification, translation, post-translational modification, secretion, fermentation, proteolysis, down-stream processing, recovery, and purification.

The mutant cell may be a mutant cell, for example, with improved production of a specific polypeptide or metabolite or a mutant cell which is no longer capable or has a
20 diminished capability of producing a specific polypeptide or metabolite. Furthermore, the mutant cell may be a mutant cell having an increased uptake of an inorganic cofactor.

The mutant cell may also have a more desirable phenotype than the parent cell which modifies the production of a polypeptide or a metabolite. The term "phenotype" is defined herein as an observable or outward characteristic of a cell determined by its genotype and
25 modulated by its environment. Such a mutant cell having a desired phenotype includes, but is not limited to, a morphological mutant cell, a secretion mutant cell, an auxotrophic mutant cell, a conditional mutant, a mutant cell exhibiting an altered growth rate under desired conditions relative to the parent cell, a mutant cell resulting in the relief of overexpression mediated growth inhibition, or a mutant cell able to tolerate low oxygen conditions.

30 Furthermore, the mutant cell may be characterized as being a mutant cell exhibiting altered production of a transcriptional activator of a promoter or a cryptic intron-splicing-deficient mutant cell.

The isolation of a mutant cell may involve screening methods known in the art specific to the desired phenotype and/or the polypeptide or the metabolite of interest. In general where a desired phenotype is involved, a method specific to the desired phenotype may be used initially to identify the mutant cell, but then may be followed by a method
5 specific to the polypeptide or the metabolite.

The population of presumptive mutants obtained by introducing a nucleic acid construct into the cells of an organism to produce a mutant cell are first purified using standard plating techniques such as those used in classical mutagenesis (see, for example, Lawrence, C.W., 1991, *In* Christine Guthrie and Gerald R. Fink, editors, *Methods in*
10 *Enzymology*, Volume 194, pages 273-281, Academic Press, Inc., San Diego), single spore isolation, or enrichment techniques. The standard plating techniques are preferably conducted in combination with a means of detecting the desired phenotype and/or the polypeptide or the metabolite. Different enrichment techniques may be used for increasing the percentage of mutant cells in comparison to their wild-type or parent equivalents such as
15 (1) direct selection which utilizes growth conditions that greatly favor the growth of the mutant; (2) counterselection, which makes use of conditions that kill the parent cells; (3) physical selection, which involves unique properties of the mutant cells that enable them to be physically separated from their parent cells; and (4) direct measurements of the amount of desired substances. However, whether or not a means for identifying the mutant cell with
20 respect to the desired phenotype and/or the polypeptide or the metabolite of interest can be incorporated into the plating medium, the purified presumptive mutants may require further characterization to confirm the identity of the mutant. Examples of the methods used to further characterize and confirm the identity of the mutant are illustrated below.

A mutant with improved production of a specific polypeptide or a specific metabolite
25 may be identified by using a detection method known in the art that is specific for the polypeptide or the metabolite. Detection methods for polypeptides may include, but are not limited to, use of specific antibodies, enzymatic activity by measuring formation of an enzyme product or disappearance of an enzyme substrate, clearing zones on agar plates containing an enzyme substrate, and biological activity assays. Detection methods for
30 metabolites may include, but are not limited to, thin layer chromatography, high performance liquid chromatography, gas chromatography, mass spectroscopy, biological activity assays, bioassays, and fluorescent activating cell sorting.

In a preferred embodiment, the specifically desired mutant cell is a mutant cell with improved production of a specific polypeptide.

In another preferred embodiment, the specifically desired mutant is a mutant with improved production of a specific metabolite; more preferably an alkaloid, an amino acid, an antibiotic, a cofactor, a drug, a fatty acid, a fungicide, a herbicide, an insecticide, an organic acid, a pigment, a plastic precursor, a polyester precursor, a prosthetic group, a rodenticide, a sweetener, or a vitamin; and most preferably citric acid or lactic acid.

A prosthetic group or an organic cofactor which is a constituent of a polypeptide and/or required for biological activity may be overproduced by isolating a mutant according to the methods of the present invention. Such a mutant would be particularly important where biosynthesis of the prosthetic group or the cofactor is a rate-limiting event in the production of a polypeptide in a biologically active form, *e.g.*, a hemoprotein containing heme including, but not limited to, a cytochrome, specifically cytochrome P450, cytochrome *b*, cytochrome *c*₁, or cytochrome *c*; a globin, specifically, hemoglobin or myoglobin; an oxidoreductase, specifically a catalase, an oxidase, an oxygenase, a haloperoxidase, or a peroxidase; or any other polypeptide containing a heme as a prosthetic group.

In a more preferred embodiment, the specifically desired mutant cell is a mutant cell overproducing an adenosine phosphate, S-adenosyl-L-methionine, biocytin, biotin, coenzyme A, coenzyme Q (ubiquinone), 5'-deoxyadenosylcobalamine, a ferredoxin, a flavin coenzyme, heme, lipoic acid, a nucleoside diphosphate, a nicotinamide adenine dinucleotide, a nicotinamide adenine dinucleotide phosphate, phosphoadenosine, phosphosulfate, pyridoxal phosphate, tetrahydrofolic acid, thiamine pyrophosphate, or a thioredoxin.

In another preferred embodiment, the specifically desired mutant cell is a mutant cell characterized with an increased uptake of an inorganic cofactor. The uptake by a cell of an inorganic cofactor which is a constituent of a polypeptide and/or required for biological activity may be increased by isolating a mutant according to the methods of the present invention. Such a mutant would be particularly important where uptake of the inorganic cofactor is a rate-limiting event in the production of a polypeptide in a biologically active form. In a more preferred embodiment, the specifically desired mutant cell is a mutant cell characterized with an increased uptake of Co^{2+} , Cu^{2+} , Fe^{2+} , Fe^{3+} , K^{+} , Mg^{2+} , Mn^{2+} , Mo , Ni^{2+} , Se , or Zn^{2+} .

In a preferred embodiment, the polypeptide or the metabolite is produced by the mutant cell in an amount which is at least 20% greater, preferably at least 50%, more preferably at least 75%, more preferably at least 100%, more preferably at least 100%-1000%, even more preferably at least 200%-1000%, and most preferably at least 500%-1000% or more greater than the cell.

In another preferred embodiment, the specifically desired mutant cell is a mutant cell which is no longer capable or has a diminished capability of producing a specific polypeptide. A mutant cell which is no longer capable or has a diminished capability of producing a specific polypeptide may be identified using the same methods described above for polypeptides, but where no or diminished production is measured relative to the parent cell.

In a more preferred embodiment, the specifically desired mutant cell is a mutant cell which is no longer capable or has a diminished capability of producing a polypeptide.

In another preferred embodiment, the polypeptide is produced by the mutant cell in an amount which is at least 20%, more preferably at least 50%, even more preferably at least 75%, and most preferably 100% lower than the cell.

In another preferred embodiment, the specifically desired mutant cell is a mutant cell which is no longer capable or has a diminished capability of producing a specific metabolite. A mutant cell which is no longer capable or has a diminished capability of producing a specific metabolite may be identified using the same or similar methods described above for metabolites, but where no or diminished production is measured relative to the parent cell.

In a more preferred embodiment, the specifically desired mutant cell is a mutant cell which is no longer capable or has a diminished capability of producing a deoxysugar, a surfactant, a mycotoxin, an organic acid, a sugar alcohol, a toxic metabolite, or a toxin; and most preferably an aflatoxin, beta-exotoxin, cyclopiazonic acid, an enniatin, a fusarin, kanosamine, mannitol, oxalic acid, surfactin, a tricothecene, a zearalenol, or a zearalenone.

In another preferred embodiment, the metabolite is produced by the mutant cell in an amount which is at least 20% lower than the cell, more preferably 50%, even more preferably 75%, and most preferably 100% lower than the cell.

In another preferred embodiment, the mutant cell is a morphological mutant cell. A "morphological mutant cell" is defined herein as a mutant cell which has a desired morphology. A morphological mutant cell may be identified, for example, by using standard

plating techniques employing a growth medium which elicits the desired morphology relative to the parent cell, by microscopic examination, or by sorting vegetatively growing cells by fluorescence activated cell sorting. Such morphological mutants include, but are not limited to, a mutant characterized as having superior rheological properties, *e.g.*, a highly-branched
5 fungal mutant, a restricted colonial fungal mutant, or a highly-branched restricted colonial fungal mutant which possesses rapid growth and low viscosity growth characteristics; a mutant which possesses a filamentous form during fermentation in contrast to a pellet form; a mutant which is less "sticky" preventing the colonization of fermentor surfaces; a mutant with a predictable viscosity during the course of a fermentation; a color mutant which aids
10 in monitoring and maintaining the purity of a culture and high production of a polypeptide by the culture; a wettable cell which lacks, for example, a cell wall or structural hydrophobic protein, *e.g.*, hydrophobin; an osmotic stress-insensitive mutant which improves growth of a cell; a desiccation-insensitive mutant which improves growth of a cell; a non-spore-forming mutant which enhances the production of a polypeptide; and a non-slime-producing mutant
15 with low viscosity growth.

Preferably, the morphological mutant cell is a color mutant, a wettable mutant cell, a mutant characterized as having superior rheological properties, an osmotic stress-insensitive mutant, a desiccation-insensitive mutant, a non-spore-forming mutant, or a non-slime-producing mutant, and most preferably a highly-branched fungal mutant, a restricted colonial
20 fungal mutant, or a highly-branched restricted colonial fungal mutant.

In another preferred embodiment, the mutant cell is a secretion mutant cell. A "secretion mutant cell" is defined herein as a mutant cell which produces higher yields of one or more secreted proteins. A secretion mutant cell may be identified by using a detection method known in the art that is specific for the polypeptide and comparing the yield to one
25 or more known secreted polypeptides at the same time. Detection methods for polypeptides may include, but are not limited to, use of specific antibodies, enzymatic activity by measuring formation of an enzyme product or disappearance of an enzyme substrate, clearing zones on agar plates containing an enzyme substrate, biological activity assays, and fluorescent activating cell sorting.

30 In another preferred embodiment, the specifically desired mutant cell is an auxotrophic mutant cell. An "auxotrophic mutant cell" is defined herein as a mutant cell which has lost its ability to synthesize one or more essential metabolites or to metabolize one

or more metabolites which modifies the production of a polypeptide by the mutant cell. An auxotrophic mutant cell may be identified using standard plating techniques by growing the presumptive mutant both in the absence and presence of an essential metabolite. The auxotrophic mutant will not grow in the absence of the essential metabolite. The auxotrophic mutant can be advantageously used to selectively screen for a mutant producing a specific polypeptide of interest.

In a more preferred embodiment, the specifically desired mutant cell is an auxotrophic mutant cell unable to metabolize or synthesize one or more of an amino acid, a fatty acid, an organic acid, a pyrimidine, a purine, or a sugar; and more preferably 5-aminolevulinic acid, biotin, glucose, lactose, or maltose.

In another preferred embodiment, the specifically desired mutant cell is a conditional mutant cell. A "conditional mutant cell" is defined herein as a mutant cell which contains one or more mutations whose phenotypes are only observed under certain conditions and modifies the production of a polypeptide or a metabolite by the mutant cell. Conditional mutations can occur in virtually all genes, including those that control the steps in macromolecular synthesis, modification, and assembly into supermolecular structures. A conditional mutant cell may be identified using standard plating techniques by growing the presumptive mutant both under permissive and restrictive conditions. For example, a mutant strain which does not produce undesirable proteolytic activity under nitrogen limited conditions would be desirable compared to the parent strain which produces proteolytic activity under nitrogen limited conditions. An additional example is an alkaline pH sensitive mutant that does not grow at alkaline pH, but may have increased or decreased production of a desired polypeptide. A further example is a mutant which is unable to grow under specific growth conditions.

In a more preferred embodiment, the conditional mutant cell is a temperature-sensitive, acid pH sensitive, alkaline pH sensitive, antibiotic-resistant, antibiotic-sensitive, toxin-resistant, toxin-sensitive, virus-resistant, or paraquat-sensitive cell; and most preferably an alkaline pH sensitive mutant cell.

In another preferred embodiment, the specifically desired mutant cell is a mutant cell exhibiting an altered growth rate relative to the parent cell. A "mutant cell exhibiting an altered growth rate" is defined herein as a mutant cell which has a doubling time that is different than that of the parent cell. Such a mutant cell may be identified by comparing the

growth of the mutant cell and the parent cell under controlled fermentation conditions. Such a mutant cell may have improved fermentation characteristics like a shorter fermentation time to increase productivity, or a longer fermentation time to provide control of the oxygen demand of a culture.

5 In another preferred embodiment, the specifically desired mutant cell is a mutant cell resulting in the relief of overexpression mediated growth inhibition. A "mutant cell resulting in the relief of overexpression mediated growth inhibition" is defined herein as a mutant cell whose growth is not inhibited by the overproduction of a desired polypeptide or metabolite when grown under conditions that induce high level production of the polypeptide or the
10 metabolite. Such a mutant may be identified by standard plating techniques on plates with an inducing carbon source, *e.g.*, maltose. Mutants would be able to grow well on the inducing carbon source while the parent cells would grow poorly. Such a mutant would be useful since it is known in some cells that overexpression of a polypeptide is toxic to the cells.

15 In another preferred embodiment, the specifically desired mutant cell is a mutant cell able to tolerate low oxygen conditions. A "mutant cell able to tolerate low oxygen conditions" is defined herein as a mutant cell which is able to grow and produce a desired polypeptide or metabolite under growth conditions where the dissolved oxygen concentration is low. Such a mutant cell is particularly advantageous for fermentations where the
20 productivity of high cell densities decreases due to oxygen transfer. A low oxygen tolerant mutant is preferably detected by growing the mutant cell relative to the parent cell on a solid or in a liquid medium in the presence of low levels of oxygen.

In a more preferred embodiment, the specifically desired mutant cell is a mutant cell able to tolerate low oxygen conditions in the range of about 0 to about 50% saturation,
25 preferably about 0 to about 40% saturation, even more preferably about 0% to about 30% saturation, more preferably about 0% to about 20% saturation, most preferably about 0% to about 10% saturation, and even most preferably about 0% to about 5% saturation.

In another preferred embodiment, the specifically desired mutant cell is a signal transduction pathway mutant cell. A "signal transduction pathway mutant cell" is defined
30 herein as a mutant cell with a mutation in one or more of the genes of the pathway which modifies the production of a polypeptide encoded by a DNA sequence of interest. The term "signal transduction pathway" is defined herein as a cascade of genes encoding polypeptides

that are all required for the activation or deactivation of another single polypeptide. The pathway senses a signal and through the cascade of genes, the signal is transduced and leads to the activation or deactivation of one or more polypeptides. Such a mutant is preferably detected using a method which is specific to the desired phenotype which modifies the
5 production of a polypeptide of interest.

In a more preferred embodiment, the signal transduction pathway mutant cell is a glucose transport signal transduction pathway mutant or a pH signal transduction pathway mutant, even more preferably a mutant in which gene required for activation of *pacC* has been disrupted, and most preferably a *gluT* gene mutant or a *palB* gene mutant.

10 In another preferred embodiment, the specifically desired mutant cell is a mutant cell exhibiting altered production of a transcriptional activator of a promoter. A "mutant cell exhibiting altered production of a transcriptional activator of a promoter" is defined herein as a mutant cell with a mutation in a gene encoding a transcriptional activator which 'turns-up' or 'turns-down' a promoter of a DNA sequence encoding a polypeptide of interest.

15 Examples of such promoters in a bacterial cell are promoters of the genes of the *Bacillus amyloliquefaciens* alpha-amylase gene (*amyQ*), the *Bacillus licheniformis* alpha-amylase gene (*amyL*), the *Bacillus licheniformis* penicillinase gene (*penP*), the *Bacillus stearothermophilus* maltogenic amylase gene (*amyM*), the *Bacillus subtilis* levansucrase gene (*sacB*), the *Bacillus subtilis* *xylA* and *xylB* genes, the *E. coli* *lac* operon, the *Streptomyces*
20 *coelicolor* agarase gene (*dagA*), and the prokaryotic beta-lactamase gene (Villa-Kamaroff *et al.*, 1978, *Proceedings of the National Academy of Sciences USA* 75:3727-3731), as well as the *tac* promoter (DeBoer *et al.*, 1983, *Proceedings of the National Academy of Sciences USA* 80:21-25). Further promoters are described in "Useful proteins from recombinant bacteria" in *Scientific American*, 1980, 242:74-94; and in J. Sambrook, E.F. Fritsch, and T. Maniatus,
25 1989, *Molecular Cloning, A Laboratory Manual*, 2d edition, Cold Spring Harbor, New York.

Examples of such promoters in a filamentous fungal cell are promoters of the genes encoding *Aspergillus nidulans* acetamidase, *Aspergillus niger* acid stable alpha-amylase, *Aspergillus niger* neutral alpha-amylase, *Aspergillus awamori* or *Aspergillus niger* glucoamylase (*glaA*), *Aspergillus oryzae* alkaline protease, *Aspergillus oryzae* TAKA amylase,
30 *Aspergillus oryzae* triose phosphate isomerase, *Fusarium oxysporum* trypsin-like protease (as described in U.S. Patent No. 4,288,627, which is incorporated herein by reference), *Rhizomucor miehei* aspartic proteinase, *Rhizomucor miehei* lipase, and mutant, truncated, and

hybrid promoters thereof. Particularly preferred promoters in filamentous fungal cells are the TAKA amylase, NA2-tpi (a hybrid of the promoters from the genes encoding *Aspergillus niger* neutral alpha-amylase and *Aspergillus oryzae* triose phosphate isomerase), and *glaA* promoters.

5 Examples of such promoters in a yeast cell are promoters of the genes encoding *Saccharomyces cerevisiae* enolase (ENO-1) gene, the *Saccharomyces cerevisiae* galactokinase gene (GAL1), the *Saccharomyces cerevisiae* alcohol dehydrogenase/glyceraldehyde-3-phosphate dehydrogenase genes (ADH2/GAP), and the *Saccharomyces cerevisiae* 3-phosphoglycerate kinase gene. Other yeast promoters are described by Romanos *et al.*,
10 1992, *Yeast* 8:423-488.

Examples of such promoters in a mammalian cell are viral promoters such as those of Simian Virus 40 (SV40), Rous sarcoma virus (RSV), adenovirus, and bovine papilloma virus (BPV).

In a more preferred embodiment, the mutant cell exhibits altered production of a
15 transcriptional activator of the TAKA, TAKA/NA2, *Fusarium oxysporum* trypsin-like protease, or a glucoamylase promoter.

In another preferred embodiment, the specifically desired mutant cell is a cryptic intron-splicing-deficient mutant cell. A "cryptic intron-splicing-deficient mutant cell" is defined herein as a mutant cell which no longer recognizes and erroneously splices a cryptic
20 intron as an authentic intron during mRNA synthesis. A cryptic intron-splicing-deficient mutant cell would be particularly useful to prevent the excision or splicing of an erroneous cryptic nuclear pre-mRNA intron from a primary transcript so a biologically active substance is produced. In this situation, the cryptic intron is actually part of the coding sequence and, therefore, is not an authentic intron but incorrectly recognized as such and erroneously
25 spliced during mRNA synthesis and processed by the parent cell. The introduction of a DNA sequence encoding a heterologous polypeptide into a parent cell such as a fungal host cell, particularly a filamentous fungal host cell, may result in this type of erroneous or aberrant splicing of the coding sequence. A cryptic intron-splicing-deficient mutant cell may be identified by screening for increased production of a polypeptide encoded by a DNA
30 sequence that is known to have a cryptic intron which leads to little or no production of the polypeptide.

In another preferred embodiment, the specifically desired mutant may be a mutant which contains two or more of the mutations described above.

Identification of Mutant Cells of the Present Invention

5 The present inventors have discovered that when certain loci in a parent cell are disrupted, the resulting mutant cell has a modified production of a polypeptide. As described above, the nucleic acid construct itself can have an effect on the production of a polypeptide. For example, the nucleic acid construct may comprise one or more copies of the nucleic acid sequence encoding the polypeptide. In addition, the nucleic acid construct may comprise a
10 promoter, transcriptional activators and repressors, etc.

When the nucleic acid construct itself can have an effect on the amount of polypeptide produced, expressed, synthesized or secreted, in order to determine whether a mutant cell of the present invention has been produced, one would have to rescue the locus as described below and introduce another nucleic acid construct which does not have an effect, *e.g.*, a
15 selectable marker, at the same locus. If the mutant cell produced by introducing the other nucleic acid construct at the same locus also has an effect on the amount of polypeptide produced, expressed, synthesized or secreted, then the original mutant cell is a mutant cell of the present invention.

20 Rescue of a Locus with the Inserted Nucleic Acid Construct and Use of a Targeting Construct

The present invention further relates to methods for rescuing a locus with the inserted nucleic acid construct comprising isolating from the identified mutant cell (i) the nucleic acid construct and (ii) the 3' and 5' flanking regions of the locus of the genome where the nucleic
25 acid construct has been integrated; and identifying the 3' and 5' flanking regions of the locus.

The nucleic acid construct and flanking regions can be isolated or rescued by methods well known in the art such as cleaving with restriction enzymes and subsequent ligation and transformation of *E. coli*, inverse PCR, random primed gene walking PCR, or probing a library of the tagged mutant. The isolated nucleic acid construct with either or both the 3'
30 and 5' flanking regions is defined herein as a "targeting construct".

The targeting construct includes between 100 - 9,000 bp, preferably 200 - 9,000 bp, more preferably 500 - 7,000 bp, even more preferably 1,000 - 7,000 bp, and most preferably

1,000 - 3,000 bp upstream and/or downstream of the integration site of the nucleic acid construct.

The targeting construct of the invention may be introduced into a different cell to modify the production of a polypeptide similar or identical to or completely different from the polypeptide modified in the original cell. The other cell may be of the same or a different species or of a different genera as the original cell. If the original cell was a fungal cell, the other cell is preferably a fungal cell. If the original cell was a bacterial cell, the other cell is preferably a bacterial cell. If the original cell was a mammalian cell, the other cell is preferably a mammalian cell.

When the cell is a different cell, integration of the targeting construct preferably occurs at a target locus which is homologous to the locus sequence of the original cell from which the targeting construct was obtained, *i.e.*, identical or sufficiently similar such that the targeting sequence and cellular DNA can undergo homologous recombination to produce the desired mutation. The sequence of the targeting construct is preferably, therefore, homologous to a preselected site of the cellular chromosomal DNA with which homologous recombination is to occur. However, it will be understood by one of ordinary skill in the art that the likelihood of a targeting construct reinserting at a target locus will depend on the cell since homologous recombination frequencies range from almost 100% in the yeast *Saccharomyces cerevisiae* to as low as 1% in *Aspergillus*. The targeting construct may integrate by non-homologous recombination at a non-target locus which is not within the DNA sequence encoding the polypeptide of interest, but results in the modification of the production of the polypeptide.

Preferably, the target locus includes DNA sequences that have greater than 40% homology, preferably greater than 60% homology, more preferably greater than 70% homology, even more preferably greater than 80% homology, and most preferably greater than 90% homology with the flanking sequences of the targeting construct.

The targeting construct may contain either or both of the 3' and 5' regions depending on whether a single cross-over or a replacement is desired. Furthermore, the targeting construct may be modified to correct any aberrant events, such as rearrangements, repeats, deletions, or insertions, which occurred during the introduction and integration of the original nucleic acid construct into the cell's genome at the locus from which it was originally rescued.

The targeting construct described above may be used as is, *i.e.*, a restriction enzyme cleaved linear nucleotide sequence, or may be circularized or inserted into a suitable vector. For example, a circular plasmid or DNA fragment preferably employs a single targeting sequence. A linear plasmid or DNA fragment preferably employs two targeting sequences.

5 The targeting construct upon introduction into a cell, in which the cell comprises a DNA sequence encoding a polypeptide of interest, integrates into the genome of the cell at a target locus or at a nontarget locus, but preferably at a target locus, not within the DNA sequence encoding the polypeptide of interest. The target locus may be on the same chromosome or the same extrachromosomal element or on a different chromosome or a different

10 extrachromosomal element as that of the DNA sequence of interest. The integration modifies the production of the polypeptide or a metabolite by the mutant cell relative to the parent cell when the mutant cell and the parent cell are cultured under the same conditions. In a preferred embodiment, the targeting construct contains a selectable marker.

Optionally, the targeting construct can be introduced into a cell as two or more

15 separate fragments. In the event two fragments are used, the fragments share DNA sequence homology (overlap) at the 3' end of one fragment and the 5' end of the other, while one carries a first targeting sequence and the other carries a second targeting sequence. Upon introduction into a cell, the two fragments can undergo homologous recombination to form a single fragment with the first and second targeting sequences flanking the region of overlap

20 between the two original fragments. The product fragment is then in a form suitable for homologous recombination with the cellular target sequences. More than two fragments can be used, designed such that they will undergo homologous recombination with each other to ultimately form a product suitable for homologous recombination with the cellular target sequences.

25 Upon introduction of the targeting construct into a cell, the targeting construct may be further amplified by the inclusion of an amplifiable selectable marker gene which has the property that cells containing amplified copies of the selectable marker gene can be selected for by culturing the cells in the presence of the appropriate selectable agent.

In a specific embodiment, the targeting construct is *SphI* linearized pDSY109, *HpaI* linearized pDSY112, *AsnI/PvuI* linearized pMT1936, *NdeI* linearized pDSY138, *AsnI/PvuI* linearized pDSY162, *BglII* linearized p4-8.1, *BglII* linearized p4-8.1, *NarI* linearized p7-

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14.1, *Bgl*II linearized pSMO717, *Bgl*II linearized pSMO321, *Nde*I linearized pHowB571, or *Nde*I linearized pSMO810.

In a most preferred embodiment, the nucleic acid construct is pDSY109.

In a most preferred embodiment, the nucleic acid construct is pDSY112.

5 In a most preferred embodiment, the nucleic acid construct is pMT1936.

In a most preferred embodiment, the nucleic acid construct is pDSY138.

In a most preferred embodiment, the nucleic acid construct is pDSY162.

In a most preferred embodiment, the nucleic acid construct is pDSY163.

In a most preferred embodiment, the nucleic acid construct is pDSY141.

10 In a most preferred embodiment, the nucleic acid construct is pSMO1204.

In a most preferred embodiment, the nucleic acid construct is pSMOH603.

In a most preferred embodiment, the nucleic acid construct is p4-8.1.

In a most preferred embodiment, the nucleic acid construct is p7-14.1.

In a most preferred embodiment, the nucleic acid construct is pHB220.

15 In a most preferred embodiment, the nucleic acid construct is pSMO717.

In a most preferred embodiment, the nucleic acid construct is pSMO321.

In a most preferred embodiment, the nucleic acid construct is pHowB571.

In a most preferred embodiment, the nucleic acid construct is pSMO810.

In a preferred embodiment, one or more targeting constructs are introduced into target
20 loci. In another preferred embodiment, each targeting construct modifies the production of
a different polypeptide or a different metabolite or a combination thereof, or results in
different phenotypes which modify the production of different polypeptides or different
metabolites or a combination thereof. In another preferred embodiment, two or more
targeting constructs together when introduced into target loci act additively or synergistically
25 to modify the production of a polypeptide or a metabolite.

Methods of Producing a Desired Polypeptide or Metabolite from Mutant Cells

The present invention further relates to the mutant cells with a desired phenotype as
host cells. Mutant cells selected for increased production of a desired polypeptide or
30 metabolite are cultivated in a nutrient medium suitable for production of the polypeptide or
metabolite using methods known in the art. For example, the cell may be cultivated by
shake flask cultivation, small-scale or large-scale fermentation (including continuous, batch,

fed-batch, or solid state fermentations) in laboratory or industrial fermentors performed in a suitable medium and under conditions allowing the polypeptide or metabolite to be expressed and/or isolated. The cultivation takes place in a suitable nutrient medium comprising carbon and nitrogen sources and inorganic salts, using procedures known in the art (see, *e.g.*, references for bacteria and yeast; Bennett, J.W. and LaSure, L., editors, *More Gene Manipulations in Fungi*, Academic Press, CA, 1991). Suitable media are available from commercial suppliers or may be prepared according to published compositions (*e.g.*, in catalogues of the American Type Culture Collection). If the polypeptide or metabolite is secreted into the nutrient medium, the polypeptide can be recovered directly from the medium. If the polypeptide or metabolite is not secreted, it is recovered from cell lysates.

The polypeptides and metabolites may be detected using methods known in the art that are specific for the polypeptides and metabolites such as those methods described earlier or the methods described in the Examples.

The resulting polypeptide or metabolite may be recovered by methods known in the art. For example, the polypeptide or metabolite may be recovered from the nutrient medium by conventional procedures including, but not limited to, centrifugation, filtration, extraction, spray-drying, evaporation, or precipitation.

The polypeptides and metabolites of the present invention may be purified by a variety of procedures known in the art including, but not limited to, chromatography (*e.g.*, ion exchange, affinity, hydrophobic, chromatofocusing, and size exclusion), electrophoretic procedures (*e.g.*, preparative isoelectric focusing), differential solubility (*e.g.*, ammonium sulfate precipitation), or extraction (see, *e.g.*, *Protein Purification*, J.-C. Janson and Lars Ryden, editors, VCH Publishers, New York, 1989).

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EXAMPLES

The following examples are put forth so as to provide those of ordinary skill in the art with a complete disclosure and description of how to make and use various constructs and perform the various methods of the present invention and are not intended to limit the scope of what the inventors regard as their invention. Unless indicated otherwise, parts are parts by weight, temperature is in degrees centigrade, and pressure is at or near atmospheric pressure. Efforts have been made to ensure accuracy with respect to numbers used (*e.g.*,

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length of DNA sequences, molecular weights, amounts, particular components, etc.), but some deviations should be accounted for.

Example 1: Strains and materials

5 The starting strains were *pyrG*-minus *Aspergillus oryzae* HowB425, *pyrG*-minus *Aspergillus oryzae* HowB101, *Aspergillus oryzae* JaL250, *Aspergillus niger* strain JRoy3 (*pyrG*Δ), *E. coli* DH5α (GIBCO-BRL, Gaithersburg, MD), and *E. coli* HB101 (GIBCO-BRL, Gaithersburg, MD).

10 PDA plates contained 39 g/l Potato Dextrose Agar (Difco) and were supplemented with 10 mM uridine for *pyrG* auxotrophs unless otherwise indicated.

 MY25 medium at pH 6.5 was composed per liter of 25 g of maltose, 2.0 g of MgSO₄·7H₂O, 10 g of KH₂PO₄, 2.0 g of citric acid, 10 g of yeast extract, 2.0 g of K₂SO₄, 2.0 g of urea, and 0.5 ml of trace metals solution. MY25 shake-flask medium was diluted 1:100 or 1:1000 with glass distilled water for use in microtiter growth experiments
15 (MY25/100 or MY25/1000). Cultures were grown at 34°C.

 2X MY Salts pH 6.5 solution was composed per liter of 4 g of MgSO₄·7H₂O, 4 g of K₂SO₄, 20 g of KH₂PO₄, 4 g of citric acid, 1 ml of trace metals, and 2 ml of CaCl₂·2H₂O (100 g/l stock solution).

 Minimal medium transformation plates were composed per liter of 6 g of NaNO₃,
20 0.52 g of KCl, 1.52 g of KH₂PO₄, 1 ml of trace metals solution, 1 g of glucose, 500 mg of MgSO₄·7H₂O, 342.3 g of sucrose and 20 g of Noble agar per liter (pH 6.5). Minimal medium transfer plates (pH 6.5) were composed per liter of 6 g of NaNO₃, 0.52 g of KCl, 1.52 g of KH₂PO₄, 1 ml of trace elements, 1 g of glucose, 500 mg of MgSO₄·7H₂O, and 20 g Noble agar.

25 The trace metals solution (1000X) was composed per liter of 22 g of ZnSO₄·7H₂O, 11 g of H₃BO₃, 5 g of MnCl₂·4H₂O, 5 g of FeSO₄·7H₂O, 1.6 g of CoCl₂·5H₂O, 1.6 g of (NH₄)₆Mo₇O₂₄, and 50 g of Na₄EDTA.

 COVE plates were composed per liter of 343.3 g of sucrose, 20 ml of COVE salts solution, 10 ml of 1 M acetamide, 10 ml of 3 M CsCl, and 25 g of Nobel agar. The COVE
30 salts (50X) solution was comprised of 26 g of KCl, 26 g of MgSO₄·7H₂O, 76 g of KH₂PO₄, and 50 ml of COVE trace metals solution. COVE trace metals solution was composed of

(per liter): 0.04 g of $\text{NaB}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$, 0.040 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 0.70 g of $\text{FeSO}_4 \cdot \text{H}_2\text{O}$, 0.80 g of $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, and 10 g of ZnSO_4 .

YEG medium was composed per liter of 5 g yeast extract and 20 g dextrose.

CM-1 agar plates at pH 6.5 were composed per liter of 0.25 g of NaCl, 0.5 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.9 g of K_2HPO_4 , 3.6 g of KH_2PO_4 , 0.1 ml of trace metals solution, 30 g of Bacto agar (Difco), pH 6.5. 11 ml of 10% urea, and 67 ml of 30% maltose.

CD medium was composed per liter of 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of K_2SO_4 , 15 g of KH_2PO_4 , 0.25 ml of trace metals solution, 0.7 g of yeast extract (Difco), 20 g of beta-cyclodextrin (Sigma C-4767). 3 ml of 50% urea, and 2 ml of 15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

10 G1-gly medium was composed per liter of 18 g of yeast extract (Difco), 80 g of 75% glycerol, and 0.5 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$.

OL-1 medium (pH 7.0) was composed per liter of 15 g of KH_2PO_4 , 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of K_2SO_4 , 0.25 ml of trace metals solution, 0.3 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (autoclaved separately), 2 g of Difco yeast extract (Difco), 0.5 g of urea (autoclaved separately), and 10 g of glucose.

OL-6 medium (pH 7.0) was composed per liter of 15 g of KH_2PO_4 , 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g of K_2SO_4 , 0.25 ml of trace metals solution, 0.3 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (autoclaved separately), 2 g of Difco yeast extract (Difco), 3 g of urea (autoclaved separately), and 60 g of glucose.

20 YPM medium was composed of 10 g of Bactopeptone and 5 g of yeast extract dissolved in 500ml of water and autoclaved, to which 50 ml of a sterilized 20% maltose solution was added.

MTBCDUY was composed per liter of 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g of K_2SO_4 , 5 g of KH_2PO_4 , 0.013 g of urea, 0.01 g of yeast extract, 0.1 g of maltose, 4.88 g of uridine, and 0.25 ml of trace metal solution 1 adjusted to pH 6.5.

4xMTBCDUY was composed per liter of 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.3 g of K_2SO_4 , 5 g of KH_2PO_4 , 0.052 g of urea, 0.04 g of yeast extract, 0.4 g of maltose, 4.88 g of uridine, and 0.25 ml of trace metal solution 1.

MDU1B was composed per liter of 45 g of Maltodextrin MD01, 1.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1.0 g of NaCl, 2.0 g of K_2SO_4 , 12.0 g of KH_2PO_4 , 7.0 g of yeast extract, 0.5 ml of trace metal solution, and 0.1 ml of pluronic acid. The trace metal solution consisted of 13.9 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 8.45 g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 6.8 g of ZnCl_2 , 2.5 g of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$, 2.5 g of

NiCl₂·6H₂O, and 3 g of citric acid. The pH of the shake flask medium was adjusted to 5.0 before being autoclaved.

1/5MDU2BP was composed per liter of 9 g of maltose, 0.2 g of MgSO₄·7H₂O, 0.4 g of K₂SO₄, 0.2 g of NaCl, 2.4 g of KH₂PO₄, 1.0 g of urea, 1.4 g of yeast extract, and 0.1 ml of trace metal solution 1.

Trace metal solution 1 was composed per liter of 13.8 g of FeSO₄·7H₂O, 8.5 g of MnSO₄·H₂O, 14.3 g of ZnSO₄·7H₂O, 2.5 g of CuSO₄·5H₂O, 0.5 g of NiCl₂·6H₂O, and 3.0 g of citric acid.

YPG plates was composed per liter of 4.0 g yeast extract, 1.0 g of K₂HPO₄, 0.5 g of MgSO₄·7H₂O, 15.0 g of dextrose, and 20.0 g of agar.

Example 2: Construction of *Aspergillus oryzae* HowB430

Aspergillus oryzae HowB430 was constructed to contain a lipase gene from *Humicola lanuginosa* (LIPOLASE™ gene, Novo Nordisk A/S, Bagsværd, Denmark).

pBANe8 was constructed as described below to contain the TAKA/NA2-tpi leader hybrid promoter, the lipase gene from *Humicola lanuginosa*, the AMG terminator, and the full-length *Aspergillus nidulans amdS* gene as a selectable marker.

PCR was employed to insert *Nsi*I sites flanking the full-length *amdS* gene of pToC90 (Christensen *et al.*, 1988, *Biotechnology* 6: 1419-1422) using primers 1 and 2 below and to insert an *Eco*RI site at the 5' end and a *Swa*I site at the 3' end of the NA2-tpi leader hybrid promoter of pJaL292 (Figure 1) using primers 3 and 4 below. The primers were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer (Applied Biosystems, Inc., Foster City, CA) according to the manufacturer's instructions.

Primer 1: 5'-ATGCATCTGGAAACGCAACCCTGA-3' (SEQ ID NO:1)

Primer 2: 5'-ATGCATTCTACGCCAGGACCGAGC-3' (SEQ ID NO:2)

Primer 3: 5'-TGGTGTACAGGGGCATAAAAT-3' (SEQ ID NO:3)

Primer 4: 5'-ATTTAAATCCAGTTGTGTATATAGAGGATTGTGG-3' (SEQ ID NO:4)

Amplification reactions (100 µl) were prepared using approximately 0.2 µg of either pToC90 or pJaL292 as the template. Each reaction contained the following components: 0.2 µg of plasmid DNA, 48.4 pmol of the forward primer, 48.4 pmol of the reverse primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Corp., Branchburg, NJ). The reactions were incubated in an

Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes.

The PCR products were electrophoresed on a 1% agarose gel to confirm the presence of a 2.7 kb *amdS* fragment and a 0.6 kb NA2-tpi fragment.

5 The PCR products were subsequently subcloned into pCRII using a TA Cloning Kit (Invitrogen, San Diego, CA) according to the manufacturer's instructions. The transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit (Qiagen, Inc., Chatsworth, CA) according to the manufacturer's instructions, and restriction digesting the plasmid DNA with either *NsiI* or *EcoRI/SwaI* followed by
10 agarose electrophoresis to confirm the presence of the correct size fragments, 2.7 kb and 0.6 kb, respectively, for the *NsiI amdS* fragment and *SwaI/EcoRI* NA2-tpi fragment. In order to confirm the PCR products, the products were sequenced with with an Applied Biosystems Model 373A Automated DNA Sequencer (Applied Biosystems, Inc., Foster City, CA) on both strands using the primer walking technique with dye-terminator chemistry (Giesecke *et al.*, 1992, *Journal of Virol. Methods* 38: 47-60) using the M13 reverse (-48) and M13
15 forward (-20) primers (New England Biolabs, Beverly, MA) and primers unique to the DNA being sequenced. The plasmids from the correct transformants were then digested with the restriction enzymes for which the plasmids were designed, separated on a 1% agarose gel, and purified using a FMC SpinBind Kit (FMC, Rockland, ME) according to the
20 manufacturer's instructions.

pKS6 (Figure 2), which contains the TAKA promoter, a polylinker, the AMG terminator, and the *Aspergillus nidulans pyrG* gene, was digested with *EcoRI* and *SwaI* to remove a portion of the TAKA promoter. This region was replaced with the NA2-tpi PCR product to produce pBANe13 (Figure 3).

25 pBANe13 was digested with *NsiI* to remove the *Aspergillus nidulans pyrG* gene. This region was then replaced with the full length *amdS* gene PCR product described above to produce pBANe6 (Figure 4).

PCR was used to insert *SwaI* and *PacI* flanking sites on the full-length *Humicola lanuginosa* lipase gene of pMHan37 (Figure 5) using primers 5 and 6 below. Primers 5 and
30 6 were synthesized as described above.

Primer 5: 5'-ATTTAAATGATGAGGAGCTCCCTTGTGCTG-3' (SEQ ID NO:5)

Primer 6: 5'-TTAATTAAGTAGAGTCGACCCAGCCGCGC-3' (SEQ ID NO:6)

The amplification reaction (100 μ l) contained the following components: 0.2 μ g of pMHan37, 48.4 pmol of primer 5, 48.4 pmol of primer 6, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase. The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute, and 72°C for 2 minutes. Two μ l of the reaction was electrophoresed on an agarose gel to confirm the amplification of the lipase gene product of approximately 900 bp.

The PCR amplified lipase gene product was then subcloned into pCRII using a TA Cloning Kit. The transformants were screened by extracting plasmid DNA from the 10 transformants using a QIAwell-8 Plasmid Kit, restriction digesting the plasmid DNA with *SwaI/PacI*, and sequencing the DNA according to the method described above to confirm the PCR product.

The lipase gene was excised from the pCRII plasmid by digesting with *SwaI* and *PacI* and subsequently subcloned into *SwaI/PacI* digested pBANe6 to produce pBANe8 (Figure 15 6).

pBANe8 was digested with *PmeI* and the linear *PmeI* fragment containing the NA2-tpi promoter, the lipase gene from *Humicola lanuginosa*, and the AMG terminator was isolated by preparative agarose electrophoresis using 40 mM Tris-acetate-1 mM disodium EDTA (TAE) buffer.

20 *Aspergillus oryzae* HowB430 was generated by transformation of *Aspergillus oryzae* HowB425 with the linear *PmeI* fragment according to the following procedure.

Aspergillus oryzae HowB425 was grown in 100 ml of 1% yeast extract-2% peptone-1% glucose at 32°C for 16-18 hours with agitation at 150 rpm. The mycelia were recovered by filtration through a 0.45 μ m filter until approximately 10 ml remained on the filter, 25 washed with 25 ml of 1.0-1.2 M $MgSO_4$ -10 mM sodium phosphate pH 6.5, filtered as before, washed again as before until 10 ml remained, and then resuspended in 10 ml of 5 mg/ml NOVOZYME 234™ (Novo Nordisk A/S, Bagsværd, Denmark) in 1.2 M $MgSO_4$ -10 mM sodium phosphate pH 6.5 (0.45 μ m filtered) in a 125 ml Ehrlenmeyer flask. The suspension was incubated with gentle agitation at 50 rpm for approximately one hour at 37°C 30 to generate protoplasts. A volume of 10 ml of the protoplast/mycelia preparation was added to a 30 ml Corex centrifuge tube, overlaid with 5 ml of 0.6 M sorbitol-10 mM Tris-HCl pH 7.5, and centrifuged at 3600 x g for 15 minutes in a swinging bucket rotor to recover the

protoplasts. The protoplasts were recovered from the buffer interface with a Pasteur pipet. The protoplasts were then washed with five volumes of STC, centrifuged, and then rewashed and centrifuged as before. The protoplasts were resuspended in STC to a final concentration of 2×10^7 protoplasts per ml.

- 5 Transformation of *Aspergillus oryzae* HowB425 for *amdS* selection was conducted with protoplasts at a concentration of 2×10^7 protoplasts per ml. Ten μg of DNA were added to 100 μl of protoplasts. A volume of 250 μl of PEG solution (60% PEG 4000-10 mM CaCl_2 -10 mM Tris-HCl pH 8.0) was then added and the mixture was placed at 37°C for 30 minutes. Three ml of 1 M sorbitol-10 mM CaCl_2 -10 mM Tris pH 7.5 (STC) was added and
- 10 the mixture was plated on Cove plates supplemented with 10 mM uridine selecting for *amdS*. The plates were incubated 7-10 days at 34°C . Transformants were transferred to plates of the same medium and incubated 3-5 days at 37°C . The transformants were purified by streaking spores and picking isolated colonies using the same plates of the same medium without sucrose under the same conditions.

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Example 3: Construction of *Aspergillus oryzae* HowB427

Aspergillus oryzae HowB425 was co-transformed with pMHan37 and pSO2 (Figure 7) to construct *Aspergillus oryzae* HowB427 to contain the lipase gene from *Humicola lanuginosa* behind the TAKA promoter.

- 20 pSO2 (Figure 7) was constructed from a genomic library of *Aspergillus oryzae* 1560. The genomic library of *Aspergillus oryzae* 1560 was constructed by first partially digesting *Aspergillus oryzae* 1560 genomic DNA with *Sau*3A (New England Biolabs, Beverly, MA). Four units of *Sau*3A were used to digest 10 μg of *Aspergillus oryzae* 1560 genomic DNA using conditions recommended by the manufacturer. The reaction was carried out at 65°C ,
- 25 and samples were taken at 5 minute intervals (from 0 to 50 minutes). The reactions were placed on ice and stopped by the addition of EDTA to 10 mM. These digests were then run on a 1% agarose gel with ethidium bromide, and the region of the gel containing DNA from 3 kb to 9 kb was excised. The DNA was then purified from the gel slice using Beta-Agarase I using a protocol provided by the manufacturer (New England Biolabs, Beverly, MA). The
- 30 size-selected DNA was then ligated into EMBL 4 arms according to the manufacturer's instructions (Clontech, Palo Alto, CA) at 16°C overnight using conditions recommended by the manufacturer. The ligation reaction was packaged and titered using a Gigapack II

Packaging Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocol. A total of 16,000 recombinant plaques were obtained, and the library was amplified using a protocol provided by the manufacturer.

Appropriate dilutions of the genomic library were made to obtain 7000 plaques per 5 150 mm petri plate as described in the protocols provided with the EMBL 4 arms. The plaques were lifted to Hybond-N plus circular filters (Amersham, Cleveland, OH) using standard protocols (Sambrook *et al.*, 1989, *supra*). The filters were fixed using UV crosslinking, and prehybridized at 42°C (5XSSPE, 35% formamide). The genomic library was probed at low stringency (35% formamide, 5XSSPE at 42°C) with a 500 bp fragment 10 consisting of the *Aspergillus niger pyrG* gene which was labeled with ³²P using a random prime DNA labeling kit (Boehringer Mannheim, Indianapolis, IN). A 3.8 kb *HindIII* fragment was isolated from one phage and subcloned into a pUC118 cloning vector to produce pSO2.

The co-transformation of *Aspergillus oryzae* HowB425 was conducted using the 15 procedure described in Example 2 except selection was on Minimal medium transformation plates. Transformants were transferred to Minimal medium transfer plates and incubated 3-5 days at 37°C. The transformants were then purified by streaking spores and picking isolated colonies using the same transfer plates under the same conditions.

20 **Example 4: Construction of plasmids pSO122, pDSY81, and pDSY82**

pSO122 was constructed as described below to contain a 1.5 kb fragment of the *Aspergillus oryzae pyrG* gene.

PCR was used to generate pSO122 by introducing a *Bam*HI restriction site at the 5' end of the *pyrG* gene of pSO2 using primers 7 and 8 shown below. Primers 7 and 8 were 25 synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

Primer 7: 5'-GCGGGATCCCTAGAGTAGGGGGTGGTGG-3' (SEQ ID NO:7)

Primer 8: 5'-GCGGGATCCCCCTAAGGATAGGCCCTA-3' (SEQ ID NO:8)

The amplification reaction (50 µl) contained the following components: 2 ng of 30 pSO2, 48.4 pmoles of the forward primer, 48.4 pmoles of the reverse primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase (Perkin-Elmer Corp., Branchburg, NJ). The reaction was incubated in an

Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. The PCR product was isolated by electrophoresis on a 1% agarose gel.

The isolated PCR product was digested with *Bam*HI and cloned into the *Bam*HI site of pBluescript SK- (Stratagene, La Jolla, CA) to yield pSO122 (Figure 8). The only homology between the genome of *Aspergillus oryzae* HowB430 and pSO122 was in the 5' end of the *pyrG* insert since the rest of the *pyrG* fragment was deleted from *Aspergillus oryzae* HowB430 as described in Example 2.

In order to reduce the frequency of targeting to this homologous region in the genome and since pSO122 contains two *Bam*HI sites, two derivatives of pSO122, pDSY81 and pDSY82 (Figure 8), were constructed in which one of the *Bam*HI sites was destroyed. The plasmids pDSY81 and pDSY82 were constructed by partially digesting pSO122 with *Bam*HI, filling-in the 5' overhangs with the Klenow fragment, closing down the plasmid by ligation and subsequent transformation into *E. coli* DH5 α (Sambrook *et al.*, 1989, *supra*). The transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit and restriction digesting the plasmid DNA with *Bam*HI to determine if one of the *Bam*HI sites had been destroyed. Plasmids with one of the *Bam*HI sites destroyed were digested with *Nsi*I/*Bam*HI to determine which *Bam*HI site had been destroyed.

Example 5: *Aspergillus oryzae* HowB430 transformation with pSO122, pDSY81, or pDSY82

Protoplasts of *Aspergillus oryzae* HowB430 were prepared as described in Example 2. A 5-15 μ l aliquot of DNA (circular pSO122, pDSY81 linearized with 4 to 12 U of *Eco*RI, or pDSY82 linearized with 15 U of *Bam*HI) was added to 0.1 ml of the protoplasts at a concentration of 2×10^7 protoplasts per ml in a 14 ml Falcon polypropylene tube followed by 250 μ l of 60% PEG 4000-10 mM CaCl₂-10 mM Tris-HCl pH 7, gently mixed, and incubated at 37°C for 30 minutes. The transformations were made either with 5 μ g of circular pSO122, 6 μ g of linearized pDSY81, or 6 μ g of linearized pDSY82. Three ml of SPTC (1.2 M sorbitol-10 mM CaCl₂-10 mM Tris pH 8) were then added and the suspension was gently mixed. The suspension was mixed with 12 ml of molten overlay agar (1X COVE salts, 1% NZ amine, 0.8 M sucrose, 0.6% Noble agar) or 3 ml of STC medium and the

suspension was poured onto a Minimal medium plate. The plates were incubated at 37°C for 3-5 days.

The transformation frequencies of the circular pSO122 transformations ranged from about 100 to 200 transformants/ μ g. A library of $\sim$ 120,000 DNA-tagged transformants of *Aspergillus oryzae* HowB430 was obtained.

The transformation frequencies of the *Eco*RI REMI pDSY81 transformations ranged from about 60 to 100 per μ g. An *Eco*RI REMI library of $\sim$ 28,000 DNA-tagged transformants of *Aspergillus oryzae* HowB430 was generated.

The transformation frequencies of the *Bam*HI REMI pDSY82 transformations ranged from about 80 to 110 transformants/ μ g. A *Bam*HI REMI library of $\sim$ 27,000 DNA-tagged transformants of *Aspergillus oryzae* HowB430 was obtained.

*Hind*III and *Sal*I REMI libraries of *Aspergillus oryzae* HowB430 were also prepared using pDSY81 as described above.

The transformation frequencies of the *Hind*III REMI pDSY81 transformations ranged from about 80 to 120 per μ g. A *Hind*III REMI library of 35,000 DNA-tagged transformants of *Aspergillus oryzae* HowB430 was generated.

The transformation frequencies of the *Sal*I REMI pDSY81 transformations ranged from about 80 to 120 per μ g. A *Sal*I REMI library of 25,000 DNA-tagged transformants of *Aspergillus oryzae* HowB430 was generated.

The *Aspergillus oryzae* HowB430 tagged mutant library pools were designated "h" for pSO122; "e" for pDSY81 digested with *Eco*RI with subsequent transformation in the presence of *Eco*RI; "b" for pDSY82 digested with *Bam*HI with subsequent transformation in the presence of *Bam*HI; "hIII" for pDSY81 digested with *Hind*III with subsequent transformation in the presence of *Hind*III; and "s" for pDSY81 digested with *Sal*I with subsequent transformation in the presence of *Sal*I. There were 123 "h" pools, 28 "e" pools, 23 "b" pools, 55 "hIII" pools, and 25 "s" pools.

The libraries described above were pooled into groups of $\sim$ 1000 transformants and stored in 10% glycerol at -80°C.

Example 6: Characterization of integration events in "REMI" *Aspergillus oryzae* HowB430 transformants

Genomic DNA was isolated from 26 of the *EcoRI* REMI transformants ("e" pool) described in Example 5 according to the following procedure. Each transformant was grown in 5 ml of YEG medium for 24 hours at 37°C in a small Petri plate. Mycelia were then collected from each culture by filtration through Whatman filter paper No. 1 (Whatman, Springfield Mill, England) and transferred to a 1.7 ml centrifuge tube. The mycelia preparations were frozen in dry ice and dried in a SpeedVac (Savant Instruments, Inc., Farmingdale, NY) overnight at room temperature. The frozen mycelia preparations were ground to a fine powder with a speared spatula and then the ground mycelia were resuspended in 0.5 ml of lysis buffer (100 mM EDTA, 10 mM Tris pH 8.0, 1% Triton X-100, 50 mM guanidine-HCl, 200 mM NaCl). RNase was added to each preparation to a final concentration of 20 µg/ml, and the preparations were incubated at 37°C for 30 minutes. Protease K was then added to each preparation to a final concentration of 0.1 mg/ml, and the preparations were incubated at 50°C for 1 hour. The preparations were centrifuged at 13,000 x g for 15 minutes, and the supernatants were applied to QIAprep-8-well strips (Qiagen, Chatsworth, CA). The wells were washed once with 0.5 ml of PB and 0.75 ml of PE supplied by the manufacturer (Qiagen, Chatsworth, CA). After removing excess PE from each well, the DNAs were eluted from the wells in 200 µl of TE buffer (10 mM Tris-1 mM EDTA pH 7.0).

The genomic DNA was digested with either *EcoRI* to determine whether integration occurred into genomic *EcoRI* sites or *SnaBI* to determine whether or not the integration events were random throughout the genome by Southern hybridization according to the procedure described by Sambrook *et al.*, 1989, *supra*. Southern blots of the digests were probed with a 1.6 kb *NheI pyrG* fragment obtained from pSO122 (Figure 8) labeled with dioxygenin using a Genius Kit according to the manufacturer's instructions. The blot was prehybridized for 2 hours and hybridized overnight at 42°C in DIG Easy Hyb. The blot was washed and processed as recommended by the manufacturer.

The Southern blot demonstrated that in 13 of 26 transformants, *EcoRI* linearized pDSY81 integrated into an *EcoRI* site in the genome, and the distribution of the integration events appeared to be random. In 20 of the 26 transformants, only a single copy of the plasmid was integrated while in 6 of the transformants at least 2 copies were integrated at

the same locus. In order to determine if the bias (of 50%) towards integration at *EcoRI* sites was due to REMI, genomic DNA was isolated as described above from 16 *Aspergillus oryzae* HowB425 transformants, in which the *EcoRI* enzyme was heat inactivated before transformation with *EcoRI* linearized pDSY81 according to the procedure described in Example 5, and submitted to Southern blot analysis as described above. Southern analysis of these transformants demonstrated that in none of the transformants did the plasmid integrate at an *EcoRI* site in the genome.

Example 7: Lipase expression screening

10 The *Aspergillus oryzae* HowB430 tagged mutant library "h", "e", and "b" pools described in Example 5 were assayed for lipase expression.

For 96-well plate screens, MY25 medium was diluted 1000-fold using a diluent made of equal volumes of sterile water and 2X MY Salts pH 6.5 solution. For 24-well plate methods, MY25 medium was diluted 100-fold using a diluent made of equal volumes of
15 sterile water and 2X MY Salts pH 6.5 solution.

Primary 96-well plate screens involved the dilution of spores from distinct pools into MY25/1000 so that one spore on average was inoculated per well when 50 μ l of medium was dispensed into the wells. After inoculation, the 96-well plates were grown for 7 days at 34°C under static conditions. Cultures were then assayed for lipase activity as described
20 below. Mutants of interest were inoculated directly into 24-well plates containing MY25/100 and were grown for 7 days at 34°C. Cultures were then assayed for lipase activity as described below. Mutants of interest were then plated on COVE plates to produce spores, spread on PDA plates to produce single colonies, and then 4 single colonies from each isolate were tested in the 24-well plate method described above.

25 The lipase assay substrate was prepared by diluting 1:50 a p-nitrophenylbutyrate stock substrate (21 μ l of p-nitrophenylbutyrate/ml DMSO) into MC buffer (4 mM CaCl_2 -100 mM MOPS pH 7.5) immediately before use. Standard lipase (LIPOLASE™, Novo Nordisk A/S, Bagsværd, Denmark) was prepared to contain 40 LU/ml of MC buffer containing 0.02% alpha olefin sulfonate (AOS) detergent. The standard was stored at 4°C until use. Standard
30 lipase was diluted 1/40 in MC buffer just before use. Broth samples were diluted in MC buffer containing 0.02% AOS detergent and 20 μ l aliquots were dispensed to wells in 96-well plates followed by 200 μ l of diluted substrate. Using a plate reader, the absorbance at 405

nm was recorded as the difference of two readings taken at approximately 1 minute intervals. Lipase units/ml (LU/ml) were calculated relative to the lipase standard.

The results of the 96-well screen followed by the 24-well screen identified for further evaluation 53 transformants from the pSO122 transformations and 44 transformants from the pDSY81 or pDSY82 REMI transformations. These identified transformants produced higher levels of lipase than the control strains *Aspergillus oryzae* HowB427 and *Aspergillus oryzae* HowB430.

Example 8: Shake flask and fermentation evaluation

10 The highest lipase-producing DNA-tagged mutants described in Example 7 were then plated onto COVE plates to produce spores for shake flask and fermentation evaluations.

Shake flask evaluations were performed by inoculating 300-500 μ l of a spore suspension (0.02% Tween-80 plus spores from the COVE plates) into 25 ml of MY25 medium at pH 6.5 in a 125 ml shake flask. The shake flasks were incubated at 34°C for 3 15 days at 200 rpm. Samples were taken at day 2 and day 3 and lipase activity was measured as described in Example 7.

The same DNA-tagged mutants were grown in a 2 liter lab fermentor containing medium composed of Nutriose, yeast extract, $(\text{NH}_4)_2\text{HPO}_4$, $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, citric acid, K_2SO_4 , $\text{CaCl}_2 \cdot \text{H}_2\text{O}$, and trace metals solution at 34°C, pH 7, 1000-1200 rpm for 8 days. Lipase 20 activity was measured as described in Example 7.

The results obtained are shown in Table 1 below where the lipase yield of either *Aspergillus oryzae* HowB427 or *Aspergillus oryzae* HowB430 as a control is normalized to 1.0.

Table I - Lipase Expression by DNA Tagged Mutants

Strain Description	Construction	Pool	# Screened in 96-well Plates	24-well Plate Results (LU/ml)	Shake Flask Results (LU/ml)	Ferm. Results (LU/ml)
5						
HowB427	HowB425 + pMHan37	NA	NA	1.2	0.6	1.0
HowB430	HowB425 + pBAnE8	NA	NA	1.0	1.0	NA
DEBY10.3	pDSY81 + BamHI	b1	808	1.7	2.2	3.9
DEBY203.3	pDSY81 + EcoRI	e1	707	2.4	2.1	1.8
10 DEBY599.3	pDSY81 + BamHI	b18	443	1.5	2.4	4.1
DEBY932	pDSY81 + EcoRI	e21	1092	1.9	1.9	3.6
DEBY1058	pDSY81 + BamHI	b22	80	2.3	2.4	3.8
DEBY1204.3.3	pDSY81 + EcoRI	e26	1260	1.9	2.0	3.0
HINL603	pDSY81 + HindIII	hi3-7	NA	2.8	2.0	3.3
15 HowL91.1	pSO122	h32	1134	1.9	2.3	3.3
HowL214.2	pSO122	h9	861	1.9	2.3	3.0
HowL301.4	pSO122	h58	731	2.3	2.8	3.6
HowL371.3	pSO122	h92	592	1.8	2.5	3.5
HowL442.1	pSO122	h7	1095	2.3	2.6	4.5
20 HowL465.2	pSO122	h8	1003	2.3	2.2	3.2
HowL500.1	pSO122	h99	885	2.3	2.7	3.4
HowL554.1	pSO122	h120	892	1.9	2.3	3.6
HowL795.4	pSO122	h29	1029	3.0	3.8	4.9

25 As shown in Table I, the mutants produced approximately 2- to 4-fold more lipase than the control strain *Aspergillus oryzae* HowB427 and approximately 3- to 6-fold more lipase than the control strain *Aspergillus oryzae* HowB430 when grown in shake flasks. The mutants also produced approximately 2- to 5-fold more lipase than the control strain *Aspergillus oryzae* HowB427 when grown in fermentors.

30

Example 9: Rescue of plasmid DNA and flanking DNA from high lipase expressing mutants

The plasmid DNA (pSO122, pDSY81, or pDSY82) and genomic flanking loci were isolated from mutants *Aspergillus oryzae* DEBY10.3, DEBY599.3, DEBY932, DEBY1058, 35 DEBY1204.3.3, and HIN603.

Genomic DNA was isolated from mutants *Aspergillus oryzae* DEBY10.3, DEBY599.3, DEBY932, DEBY1058, DEBY1204.3.3, and HIN603 according to the following procedure. Spore stocks of each mutant were inoculated into 150 ml of YEG medium and were grown overnight at 37°C and 250 rpm. The mycelia were harvested from 40 each culture by filtration through Miracloth (Calbiochem, La Jolla, CA) and rinsed twice with TE. The mycelia preparations were then frozen quickly in liquid nitrogen and ground

to a fine powder with a mortar and pestle. The powdered mycelia preparations were each transferred to a 50 ml tube and 20 ml of lysis buffer was added. RNase was added to each preparation to a final concentration of 20 µg/ml, and the preparations were incubated at 37°C for 30 minutes. Protease K was then added to each preparation to a final concentration of 0.1 mg/ml, and the preparations were incubated at 50°C for 1 hour. The preparations were then centrifuged at 15,000 x g for 20 minutes to pellet the insoluble material. Each supernatant was applied to a Qiagen MAXI column (Qiagen, Chatsworth, CA) which was equilibrated with QBT provided by the manufacturer. The columns were then washed with 30 ml of QC provided by the manufacturer. DNA was eluted from each column with 15 ml of QF provided by the manufacturer and then recovered by precipitation with a 0.7 volume of isopropanol and centrifugation at 15,000 x g for 20 minutes. The pellets were finally washed with 5 µl of 70% ethanol, air-dried, and dissolved in 200 µl of TE.

Two µg aliquots from each of the *Aspergillus oryzae* DEBY10.3, DEBY599.3, DEBY932, DEBY1058, DEBY1204.3.3, and HIN603 genomic DNA preparations were digested separately with *Bgl*III, *Hpa*I, *Nar*I, *Nde*I, *Sph*I, and *Stu*I. The restriction endonucleases did not cut pDSY82 which allowed the isolation of the integrated plasmid and the flanking genomic DNA. The digested genomic DNAs were then ligated in a 20 µl reaction with T4 DNA ligase.

The ligated DNA preparations were each transformed into *E. coli* HB101 or *E. coli* DH5α. The transformants were then screened by extracting plasmid DNA from the transformants, restriction digesting the inserts to confirm they are derived from pDSY82, and sequencing the inserts according to the method described above using primers specific to pDSY82.

Transformant *E. coli* HB101 - pDSY112 contained the *Hpa*I rescued locus from mutant *Aspergillus oryzae* DEBY599.3. Transformant *E. coli* HB101 - pDSY109 contained the *Sph*I rescued locus from mutant *Aspergillus oryzae* DEBY10.3. Transformant *E. coli* HB101- pDSY138 contained the *Nde*I rescued locus from mutant DEBY932. Transformant *E. coli* HB101- pDSY141 contained the *Bgl*III rescued locus from mutant DEBY1058. Transformant *E. coli* DH5α - pSMO1204 contained the *Bgl*III rescued locus from mutant *Aspergillus oryzae* DEBY1204.3.3. Transformant *E. coli* DH5α - pSMOH603 contained the *Bgl*III rescued locus from mutant *Aspergillus oryzae* HIN603.

Example 10: Characterization of *Aspergillus oryzae* DEBY599.3 rescued locus pDSY112

The *Aspergillus oryzae* DEBY599.3 rescued locus pDSY112 containing 1625 bp was sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ ID NO:9) and the deduced amino acid sequence (SEQ ID NO:10) are shown in Figure 9.

- 5 The nucleic acid sequence suggested that integration occurred within the promoter of a glucose transporter about 150 bp upstream of the ATG start codon. The open reading frame was punctuated by an intron. The predicted protein (SEQ ID NO:10) shared 31.6% and 24.8% identity with the glucose transporters from yeast (SEQ ID NO:11) and human (SEQ ID NO:12), respectively, and 20.1% identity with an inositol transporter from yeast (SEQ ID NO:13). Glucose transporters have very distinct predicted secondary structures with 12 membrane spanning domains. Kyte-Doolittle plots of the *Aspergillus oryzae* DEBY599.3 rescued locus predicted 12 membrane spanning domains similar to the yeast and human glucose transporters.

- In order to confirm that the rescued flanking DNA was the gene disrupted in *Aspergillus oryzae* DEBY599.3, a Southern blot of *Aspergillus oryzae* HowB101 and *Aspergillus oryzae* DEBY599.3 genomic DNA preparations digested with *Bgl*III was prepared and analyzed according to the procedure described in Example 6. The blot was probed with the *Aspergillus oryzae* DEBY599.3 rescued flanking DNA at 42°C in DIG Easy Hyb. The blot was then washed and processed using protocols provided with a Genius Kit.

- 20 A *Bgl*III band of 2.7 kb from *Aspergillus oryzae* HowB101 hybridized with the probe, while an ~8 kb *Bgl*III band from *Aspergillus oryzae* DEBY599.3 hybridized to the probe. The size difference corresponded to the length of the plasmid integrated during REMI confirming the DNA rescued from *Aspergillus oryzae* DEBY599.3 was flanking the insertion.

25 **Example 11: *Aspergillus oryzae* transformation with *Hpa*I linearized pDSY112 and lipase expression screening**

- Aspergillus oryzae* HowB430 was transformed with *Hpa*I digested pDSY112 and the transformants were recovered using the methods described in Example 5. Totally, 216 transformants were grown in 24 well microtiter plates in 1/100 strength MY25 medium. Samples were taken at 4 and 6 days and assayed for lipase activity as described in Example 7. An equal number of low, average and high producing lipase transformants were spore purified and retested in 24 well microtiter cultures as described above. These purified

transformants were also tested in shake flasks in full-strength MY25 medium as described in Example 8. The top five producing transformants were then grown in a 2 liter fermentor as described in Example 8. Lipase activity was measured as described in Example 7.

The results obtained are shown in Table 2 below where the lipase yield of *Aspergillus oryzae* HowB430 is normalized to 1.0.

Table 2

	<u>Strain</u>	<u>Fermentation Results (Relative LU/ml)</u>
	HowB430	1.0
	DEBY599.3	1.7
10	112T90.2.2	2.3
	112T100.4.2	2.1
	112T344.2.1	1.7
	112T142.2	2.0
15	112T59.2	2.4

All five retransformants produced approximately the same level of lipase activity as the original tagged strain *Aspergillus oryzae* DEBY599.3 when grown under fermentation conditions. In order to determine if the pDSY112 had integrated at the same homologous locus in the genome, a Southern blot of *Aspergillus oryzae* HowB430, *Aspergillus oryzae* DEBY599.3 and the pDSY112 transformants genomic DNA preparations digested with *Bgl*II was prepared and analyzed according to the procedure described in Example 6. The blot was probed with the *Aspergillus oryzae* DEBY599.3 rescued flanking DNA at 42°C in DIG Easy Hyb. The blot was washed and processed using protocols provided with a Genius Kit.

A *Bgl*II band of 2.7 kb from *Aspergillus oryzae* HowB430 hybridized with the probe, while an ~8 kb *Bgl*II band from *Aspergillus oryzae* DEBY599.3 hybridized to the probe. A wild-type *Bgl*II band of 2.7 kb and a second band corresponding to the transforming DNA hybridized to the probe in all of the transformants. Therefore, none of the retransformants had exact gene replacements.

30 **Example 12: Characterization of *Aspergillus oryzae* DEBY10.3 rescued locus pDSY109**

The 3.4 and 2.2 kb regions on either side of the integration event of the *Aspergillus oryzae* DEBY10.3 rescued locus pDSY109 were sequenced according to the procedure described in Example 2. The nucleic acid sequence suggested that the integration event

occurred within the open reading frame of a *palB* gene. *palB* genes encode a cysteine protease involved in the signal transduction pathway that signals ambient pH.

The genomic library of *Aspergillus oryzae* HowB430 was constructed by first partially digesting *Aspergillus oryzae* HowB430 genomic DNA with *Tsp509I*. Four units of *Tsp509I* were used to digest 3.5 µg of *Aspergillus oryzae* HowB430 genomic DNA using conditions recommended by the manufacturer. The reaction was carried out at 65°C, and samples were taken at 5 minute intervals (from 0 to 50 minutes). The reactions were placed on ice and stopped by the addition of EDTA to 10 mM. These digests were then run on a 1% agarose gel with ethidium bromide, and the region of the gel containing DNA from 3 kb to 9 kb was excised. The DNA was then purified from the gel slice using Beta-Agarase I using a protocol provided by the manufacturer (New England Biolabs, Beverly, MA). The size-selected DNA was then ligated into Lambda ZipLox *EcoRI* arms according to the manufacturer's instructions at 16°C overnight using conditions recommended by the manufacturer. The ligation reaction was packaged and titered using a Gigapack GoldIII Packaging Kit according to the manufacturer's protocol. 8 X 10⁶ recombinant plaques were obtained, and the library was amplified using a protocol provided by the manufacturer.

The genomic library was screened to obtain a genomic clone of *palB*. Appropriate dilutions of the genomic library were made to obtain 7000 plaques per 150 mm petri plate as described in the protocols provided with the Lambda ZipLox arms. The plaques were lifted to Hybond-N plus circular filters using standard protocols (Sambrook *et al.*, 1989, *supra*). The filters were fixed using UV crosslinking, and prehybridized at 42°C in DIG Easy Hyb. The filters were hybridized with a DIG-labeled 0.25 kb *palB* probe. The probe was labeled with dioxygenin using a Genius Kit and PCR amplified with the following primers synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions:

5'-CTGCCGTCGAAGGTGTCCAAG-3' (SEQ ID NO:14)

5'-ATTGTGGCCCCTATGTGGATT-3' (SEQ ID NO:15)

The parameters for PCR are as described in Example 2. The filters were washed and processed post-hybridization using protocols provided with the Genius Kit. Several positive plaques were identified and purified to homogeneity using standard protocols (Sambrook *et al.*, 1989, *supra*).

The nucleotide sequence was determined for the *palB* gene according to the method described in Example 2. The nucleic acid sequence (SEQ ID NO:16) and the deduced amino acid sequence (SEQ ID NO:17) are shown in Figure 10. The open reading frame was interrupted by 3 introns. The *Aspergillus oryzae* PalB protein (SEQ ID NO:17) shared 5 66.4% identity with the *Aspergillus nidulans* PalB protein (SEQ ID NO:18). The site of insertion also contained a highly conserved domain of 37 amino acids (SEQ ID NO:19) similar to that derived from the *Neurospora crassa* NADH dehydrogenase (SEQ ID NO:20) which was probably a piece of mitochondrial DNA that inserted during transformation or rescue in *E. coli*.

10 A Southern blot of *Aspergillus oryzae* DEBY10.3 and *Aspergillus oryzae* HowB101 genomic DNA digested with *Bgl*II was prepared according to the emthod described in Example 6. The blot was probed with the *Aspergillus oryzae* DEBY10.3 rescued flanking DNA to confirm that the rescued flanking DNA was the gene disrupted in *Aspergillus oryzae* DEBY10.3.

15 A *Bgl*II band of ~7.5 kb from *Aspergillus oryzae* HowB101 hybridized to the probe while a band of 12 kb from *Aspergillus oryzae* DEBY10.3 hybridized to the probe. The size difference was the expected size for one plasmid copy being integrated confirming the locus rescued was disrupted in *Aspergillus oryzae* DEBY10.3.

Because the integration event in *Aspergillus oryzae* DEBY10.3 would be predicted to 20 lead to a nonfunctional PalB protein, *Aspergillus oryzae* DEBY10.3 was tested for growth at pH 8.0 and pH 6.5. *Aspergillus nidulans palB* minus strains are unable to grow at pH 8.0 but are able to grow at pH 6.5. *Aspergillus oryzae* HowB430 and *Aspergillus oryzae* DEBY10.3 were grown in Minimal medium with 10 mM uridine at either pH 8.0 or pH 6.5. As predicted, *Aspergillus oryzae* DEBY10.3 was unable to grow at pH 8.0.

25

Example 13: Construction of pMT1936

pMT1936 was constructed to contain a disruption cassette of *palB* using the following primers synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

30 100752: 5'-GGTTGCATGCTCTAGACTTCGTCACCTTATTAGCCC-3' (SEQ ID NO:21)

100753: 5'-TTCGCGCGCATCAGTCTCGAGATCGTGTGTCGCGAGTACG-3' (SEQ ID NO:22)

100754: 5'-GATCTCGAGACTAGTGCGCGCGAACAGACATCACAGGAACC-3' (SEQ ID NO:23)

100755: 5'-CAACATATGCGGCCGCGAATTCATTCCCACTGCGTGG-3' (SEQ ID NO:24)

The *Aspergillus oryzae palB* 5' flanking sequence and the sequence encoding the N-terminal part of the *palB* product were PCR amplified from genomic DNA of *Aspergillus oryzae* A1560 obtained according to the method described in Example 2. Approximately 5 0.05 μ g of DNA template and 5 pmole of each of the two primers 100755 and 100754 were used. Amplification was performed with the polymerase *Pwo* as described by the manufacturer (Boehringer Mannheim, Indianapolis, IN). Amplification proceeded through 40 cycles. Part of the reaction product was phenol extracted, ethanol precipitated, digested with restriction enzymes *EcoRI* and *XhoI* and a fragment of approximately 1.05 kb was 10 isolated by agarose gel electrophoresis.

The *Aspergillus oryzae palB* 3' flanking sequence and the sequence encoding the C-terminal part of the *palB* gene product were obtained as described above except that primers 100753 and 100752 were used for amplification and the PCR product was digested with restriction enzymes *XhoI* and *XbaI* before gel electrophoresis to recover a fragment of 15 approximately 1.50 kb.

The two digested and purified PCR fragments described above were ligated in a three part ligation with the purified 2.7 kb *EcoRI-XbaI* fragment from the vector pJaL400 (Figure 11) to produce pMT1935 (Figure 12). The *palB* 5' and 3' flanks of pMT1935 are separated by *BssHII*, *SpeI*, and *XhoI* sites introduced via PCR primers 100754 and 100753.

20 To insert an *Aspergillus oryzae pyrG* gene between the *palB* 5' flank and the 3' flank of pMT1935, the 3.5 kb *HindIII* fragment of pJaL394 (Figure 13) containing the repeat flanked *pyrG* gene was cloned into *HindIII* cut, dephosphorylated and purified pBluescript II SK (-). Plasmids with inserts in either orientation were obtained. One plasmid, pMT1931 (Figure 14), was selected in which the *SpeI* site of the pBluescript polylinker was 25 downstream of the *pyrG* gene and the *XhoI* site was upstream of the *pyrG* gene. The *pyrG* gene was isolated as a 3.5 kb *SpeI-XhoI* fragment and inserted in *SpeI* and *XhoI* digested and purified pMT1935 to produce the disruption plasmid pMT1936 (Figure 15).

The *pyrG* selectable *palB* disruption cassette can be isolated from pMT1936 as a 6.2 kb *NotI* fragment (*NotI* cutting in polylinkers) or as a 5.5 kb *AseI-PvuI* fragment (*AseI* and 30 *PvuI* cutting within the actual *palB* 5' and 3' flanking sequences).

Example 14: *Aspergillus oryzae* transformation with *AseI/PvuI palB* disruption cassette from pMT1936 and lipase screening

Aspergillus oryzae HowB430 was transformed using the same transformation procedure described in Example 5 with a 5.5 kb *AseI/PvuI* fragment obtained from pMT1936. The linear fragment for transformation was isolated by digestion of pMT1936 with *AseI* and *PvuI* and separation of the fragment on a 1% agarose gel using a QIAquick Gel Extraction Kit according to the manufacturer's instructions. The transformants were then tested for growth on Minimal medium plates at pH 6.5 or pH 8.0.

The results showed that 13 of the 128 transformants tested possessed the *palB* minus phenotype as indicated by the inability to grow at pH 8.0. The 13 *palB* minus strains and 13 of the transformants that were able to grow at pH 8.0 were spore purified and then evaluated in 24-well plate and shake flask cultures for lipase production using the methods described in Examples 7 and 8, respectively. The results are shown in Table 3 below.

Southern blots of the genomic DNA from an *Aspergillus oryzae palB* minus mutant, an *Aspergillus oryzae palB* plus strain, and *Aspergillus oryzae* HowB430 were performed to determine if the *AsnI/PvuI* transforming DNA fragment had integrated as a clean replacement into the *palB* locus. The genomic DNAs were prepared according to the procedure described in Example 9, digested with *PvuI*, and electrophoresed on a 0.8% agarose gel. The DNAs were transferred to a Hybond N⁺ membrane using 0.4 N NaOH and capillary action. The blot was UV crosslinked prior to prehybridization at 65°C in Rapid Hyb. The blot was then probed with a 0.9 kb *AsnI/SpeI* fragment from pMT1936. The 0.9 kb fragment was isolated from an agarose gel slice using QiaQuick spin column after electrophoreses on a 1% agarose gel. The fragment was labeled using Vistra ECF Random Prime Labeling Kit. The blots were prehybridized and hybridized at 65°C in Rapid Hyb (Amersham, Cleveland, OH), and then washed twice for 5 minutes in 2XSSC, 0.1% SDS at 65°C and twice for 10 minutes in 0.2X SSC, 0.1% SDS at 65°C. Following the washes, the blot was processed for detection using the Vistra ECF Signal Amplification Kit (Amersham, Cleveland, OH) and the STORM860 Imaging System (Molecular Dynamics, Sunnyvale, CA).

The Southern blot results demonstrated that the probe hybridized to a band of 6 kb from *Aspergillus oryzae* HowB430. A clean disruption would be expected to hybridize to about an 8 kb *PvuI* band. The Southern blot results further showed that some of the *palB*

minus strains had clean disruptions while others did not. The Southern blot results are summarized in Table 3.

Three of the *palB* minus strains were also run under fermentation conditions according to the procedure described in Example 8. The results obtained are shown in Table 3 below where the lipase yield of *Aspergillus oryzae* HowB430 is normalized to 1.0. The three *palB* minus strains performed better or close to the same as the original tagged mutant *Aspergillus oryzae* DEBY10.3.

Table 3

		PalB	24 well	Shake flasks	Fermentation	
	<u>Strain</u>	<u>phenotype</u>	<u>LU/ml</u>	<u>LU/ml</u>	<u>results</u>	<u>Southern</u>
					<u>LU/ml</u>	<u>pattern</u>
10	HowB430	plus	1.0	1.0	1.0	wild type
	palB3-1	plus	1.2	1.1	1.7	wild-type
	palB4-1	plus	1.0	0.8	1.4	and other
	palB5-1	minus	1.4	1.4	2.0	wild-type
15	palB8-1	plus	0.9	1.0	NA	and other
	palB18-1	plus	0.9	NA	NA	wild-type
	palB27-1	plus	1.0	NA	NA	and other
	palB29-1	minus	0.8	NA	NA	wild-type
20	palB30-1	plus	0.9	NA	1.0	and other
	palB31-1	minus	1.3	NA	NA	other
	palB37-1	plus	0.8	NA	NA	wild-type
	palB39-1	plus	0.9	NA	NA	and other
	palB41-1	plus	1.0	0.8	NA	wild-type
25	palB42-1	plus	1.2	1.0	NA	and other
	palB43-1	minus	1.3	1.2	NA	wild-type
	palB69-1	plus	1.2	1.4	NA	and other
	palB71-1	minus	1.2	1.3	1.8	other
	palB72-1	minus	1.5	1.6	2.0	other
30	palB75-1	minus	1.3	1.4	1.3	other
	palB76-1	minus	1.6	1.3	2.0	clean
						disruption

palB79-1	plus	1.2	1.0	NA	wild-type and other
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Example 15: Characterization of *Aspergillus oryzae* DEBY932 rescued locus pDSY138

The *Aspergillus oryzae* DEBY932 rescued locus pDSY138 containing 1625 bp was
 5 sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ
 ID NO:25) and deduced amino acid sequence (SEQ ID NO:26) are shown in Figure 16. The
 nucleic acid sequence showed that the *EcoRI* site of the REMI integration was 810 bp
 upstream of the ATG start codon for an open reading frame and the deduced amino acid
 sequence (SEQ ID NO:26) had significant identity to mannitol-1-phosphate dehydrogenases
 10 from *E. coli* and *Bacillus subtilis*. The open reading frame coded for a predicted protein of
 319 amino acids, and shared 13.3% and 34.7% identity with the *E. coli* (SEQ ID NO:27)
 and the *Bacillus subtilis* (SEQ ID NO:28) mannitol-1-phosphate dehydrogenases, respectively.

A Southern blot of *Aspergillus oryzae* DEBY932 and *Aspergillus oryzae* HowB430
 genomic DNA preparations digested with *NdeI* was prepared and analyzed according to the
 15 method described in Example 14. The blot was probed with the *Aspergillus oryzae*
 DEBY932 rescued flanking DNA to confirm that the rescued flanking DNA is the gene
 disrupted in DEBY932.

An *NdeI* band of approximately 5 kb from *Aspergillus oryzae* HowB430 hybridized
 to the rescued locus while a band of approximately 10 kb from *Aspergillus oryzae* DEBY932
 20 hybridized to the probe confirming that the rescued locus was the disrupted locus in
Aspergillus oryzae DEBY932.

**Example 16: *Aspergillus oryzae* transformation with *NdeI* linearized pDSY138 and lipase
 expression screening**

25 *Aspergillus oryzae* HowB430 was transformed with *NdeI* digested pDSY138 and the
 transformants were recovered using the methods described in Example 5. Totally, 180
 recovered transformants were grown in 24 well microtiter plates in 1/100 strength MY25,
 and samples were taken at 4 and 6 days for lipase assays as described in Example 7. The
 top 11 highest lipase producing and 1 average lipase producing transformants were spore
 30 purified and retested in 24 well microtiter cultures. These purified transformants were also
 evaluated in shake flasks in full-strength MY25 as described in Example 8. The top two

producers were also grown in a 2 liter fermentor as described in Example 8. Lipase activity was measured as described in Example 7.

The results obtained are shown in Table 4 below where the lipase yield of *Aspergillus oryzae* HowB430 was normalized to 1.0. The top two lipase producers produced essentially the same amount of lipase activity as the original tagged mutant *Aspergillus oryzae* DEBY932.

Table 4
Fermentation Results

	<u>Strain</u>	<u>(Relative LU/ml)</u>	<u>Southern Results</u>
10	HowB430	1.0	Wild-type
	DEBY932.3.3	2.1	Disrupted
	138T83.1.1	2.2	Disrupted
	138T102.1.1	1.9	Disrupted

15 A Southern blot of *Aspergillus oryzae* DEBY932, *Aspergillus oryzae* HowB430 and pDSY138 genomic DNA preparations digested with *NdeI* was prepared and analyzed as described in Example 14 to determine if pDSY138 had integrated at the homologous locus producing gene replacements in the transformants using the *Aspergillus oryzae* DEBY932 rescued flanking DNA as a probe.

20 The Southern blot showed that an *NdeI* band of approximately 5 kb from *Aspergillus oryzae* HowB430 hybridized to the rescued locus while a band of approximately 10 kb from *Aspergillus oryzae* DEBY932 hybridized to the probe. In Table 4, the column labeled Southern results indicated whether the transformants had a wild-type *NdeI* fragment of the size observed in the parent strain *Aspergillus oryzae* HowB430 or whether the transformants
25 had a band corresponding to the disrupted size observed in *Aspergillus oryzae* DEBY932.

Example 17: Characterization of *Aspergillus oryzae* DEBY1058 rescued locus pDSY141

The *Aspergillus oryzae* DEBY1058 rescued locus pDSY141 containing approximately 1 kb was sequenced according to the method described in Example 2. The nucleic acid
30 sequence demonstrated that the rescued locus contained flanking DNA from only one side of the *BamHI* REMI integration event, and the pDSY141 sequence had rearranged.

A Southern blot of *Aspergillus oryzae* DEBY1058 genomic DNA digested with *BamHI* was probed with the *Aspergillus oryzae* DEBY1058 rescued flanking DNA was prepared and

analyzed as described in Example 14 to confirm that the rescued flanking DNA is the gene disrupted in *Aspergillus oryzae* DEBY1058.

The Southern analysis showed that the pDSY82 DNA had integrated as a REMI event at a *Bam*HI site, but more than one copy of pDSY82 had integrated which suggested why the rescued plasmid had rearranged and only contained one side of the flanking DNA.

In order to obtain the other flanking piece, a genomic clone (pDSY163) was isolated from the *Aspergillus oryzae* HowB430 genomic library, prepared as described in Example 6, using a ³²P-labeled 0.5 kb fragment of the rescued genomic DNA from *Aspergillus oryzae* DEBY1058. The probe was labeled using a Prime-It Kit according to the manufacturer's instructions (Stratagene, La Jolla, CA). Five plates of approximately 7000 plaques each were plated, and the plaques were lifted to Hybond-N⁺ as described in Example 12. The filters were prehybridized at 42°C in 50% formamide, 5XSSPE, 0.3% SDS and 200 µg/ml of sheared and denatured salmon sperm DNA for 1 hour. The denatured probe was added, and the filters were hybridized overnight at 42°C. The filters were washed in 1XSSC, 0.1% SDS for 5 minutes at 65°C twice, in 0.1XSSC, 0.1% SDS at 65°C for 15 minutes twice, and in 2XSSC at room temperature for 10 minutes. The filters were exposed to X-ray film, and 12 positive plaques were picked and purified using standard protocols (Sambrook *et al.*, 1989, *supra*). Plasmid DNA was isolated from the purified genomic clones using the excision protocol provided with the Lambda ZipLox *Eco*RI Arms Kit.

The nucleotide sequence of 3.6 kb of the genomic clone was determined as described in Example 2. The nucleic acid sequence (SEQ ID NO:29) and deduced amino acid sequence (SEQ ID NO:30) are shown in Figure 17. The nucleic acid sequence showed that the *Bam*HI site of integration in the mutant is 250 bp downstream of the stop codon for an open reading frame that encodes a protein (SEQ ID NO:30) which shared significant identity with manganese superoxide dismutase from *Saccharomyces cerevisiae* (SEQ ID NO:31).

Since the site of integration in *Aspergillus oryzae* DEBY1058 was 250 bp downstream of the stop codon for the manganese superoxide dismutase gene, the effect of this integration on expression of the manganese superoxide dismutase was determined. *Saccharomyces cerevisiae* strains lacking a functional manganese superoxide dismutase are sensitive to paraquat when grown in the presence of oxygen. *Aspergillus oryzae* DEBY1058 and *Aspergillus oryzae* HowB430 were grown in 24 well microtiter plates in 1 ml of YEG medium supplemented with 10 mM uridine and either 0, 2, 4, 6, 8, 10 or 20 mM paraquat

at 34°C with shaking. *Aspergillus oryzae* HowB430 grew at concentrations of paraquat up to 8 mM while growth of *Aspergillus oryzae* DEBY1058 was inhibited by 2 mM paraquat. The data indicated that the integration event 250 bp downstream of the stop codon for manganese superoxide dismutase in *Aspergillus oryzae* DEBY1058 reduced expression of
5 manganese superoxide dismutase.

Example 18: Construction of pDSY162

pDSY162 was constructed to contain a disruption cassette for manganese superoxide dismutase by PCR amplification of a 3179 bp *XbaI/KpnI* fragment of genomic DNA
10 containing the manganese superoxide dismutase gene using the following primers synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

970738: 5'-GCTCTAGATCGTCGGAGCTCATGTCGGCGATTTTAC-3' (SEQ ID NO:32)

970739: 5'-GCGGTACCACGCCTAGAGCAAAGTATAAATAAGGAA-3' (SEQ ID NO:33)

15 The amplification reaction (100 µl) contained the following components: 0.2 µg of the pDSY163, 48.4 pmol of primer 970738, 48.4 pmol of primer 979739, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase. The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1
20 minute, and 72°C for 2 minutes. Two µl of the reaction were electrophoresed on an agarose gel to confirm the amplification of the PCR product of approximately 3179 bp.

The PCR product was subcloned into pCR®TOPO using a TOPO TA Cloning Kit (Invitrogen, San Diego, CA). The transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit according to the manufacturer's
25 instructions, restriction digesting the plasmid DNA using *XbaI/KpnI* to confirm the presence of the correct size fragment, and sequencing the DNA according to the method described in Example 2 to confirm the PCR product.

The plasmids containing the manganese superoxide dismutase insert were digested with *XbaI* and *KpnI* and separated on a 1% agarose gel. A 3.1 kb manganese superoxide
30 dismutase fragment was purified using a QIAquick Gel Extraction Kit according to the manufacturer's instructions. The purified fragment was ligated with pBluescript SK- digested

with *Xba*I and *Kpn*I to produce pDSY161 (Figure 18). The ligation reaction was used to transform *E. coli* DH5 α .

The transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit according to the manufacturer's instructions and
5 digesting the plasmids with *Hind*III to determine which clones were correct.

pDSY161 was digested with *Hind*III to remove a 600 bp fragment, and the digestion was electrophoresed on a 1% agarose gel. A 5.4 kb vector fragment was isolated using a QIAquick Gel Extraction Kit according to the manufacturer's instructions, and ligated to the 3.5 kb *Hind*III fragment from pJaL394 (Figure 13) containing a *pyrG* gene repeat to produce
10 pDSY162 (Figure 19). The ligation reaction was used to transform *E. coli* DH5 α .

The transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit according to the manufacturer's instructions and digesting them with *Hind*III to determine which plasmids contained the expected 3.5 kb *Hind*III fragment in pDSY162.

15

Example 19: *Aspergillus oryzae* transformation with *Asn*I/*Pvu*I manganese superoxide dismutase disruption cassette and lipase screening

Aspergillus oryzae HowB430 was transformed with a 5.8 kb *Ase*I/*Pvu*I fragment containing the manganese superoxide dismutase disruption cassette using the same
20 transformation procedure described in Example 5. The linear fragment for transformation was isolated by digestion of pDSY162 with *Ase*I and *Pvu*I and separation of the fragment on a 1% agarose gel using a QIAquick Gel Extraction Kit according to the manufacturer's instructions. The transformants were then tested for growth on Minimal medium plates at pH 6.5.

25 Six transformants were obtained and were tested for sensitivity to paraquat as described in Example 17. Four of the 6 transformants were paraquat sensitive indicative of the manganese superoxide dismutase disruption minus phenotype although the four paraquat sensitive strains were not equally sensitive to paraquat. As shown in Table 5 below, a dash means not sensitive to paraquat, ++ means sensitive means sensitive to intermediate levels
30 of paraquat and +++ means inhibited by even 2 mM paraquat. All of the transformants were spore purified and tested in 24 well and shake flask cultures for lipase production according to the procedures described in Examples 7 and 8. The results tabulated in Table

5 below show that following transformation of *Aspergillus oryzae* HowB430 with the manganese superoxide dismutase disruption cassette, transformants sensitive to paraquat on average produced higher LIPOLASE™ levels than *Aspergillus oryzae* HowB430.

Southern blots of the genomic DNA from an *Aspergillus oryzae* manganese superoxide dismutase minus mutant, an *Aspergillus oryzae* manganese superoxide dismutase plus strain, and *Aspergillus oryzae* HowB430 were performed as described in Example 14 to determine if the *AsnI*/*PvuI* transforming DNA fragment had integrated as a clean replacement into the manganese superoxide dismutase locus.

The results of the Southern blot (Table 5) showed that strains sensitive to even 2 mM paraquat were disrupted at the manganese superoxide dismutase locus while those sensitive to intermediate levels of paraquat have both a wild-type locus and the disrupted cassette locus. The Southern blot and LIPOLASE™ yield results together suggests that expression of both full length and truncated manganese superoxide dismutase in the same cell leads to an intermediate sensitivity to paraquat and an increase in LIPOLASE™ production. This can be explained by the fact that manganese superoxide dismutase is a homodimer so expression of the wild-type and truncated forms coded for by the wild-type and disrupted cassette, respectively, leads to heterodimers which are either non-functional or partially functional.

Table 5

20	Strain	Paraquat	24 well results	Shake flasks results	Southern Results
		sensitivity	(Relative LU/ml)	(Relative LU/ml)	
	HowB430	-	1.0	1.0	wild-type
	430162T1	-	1.0	1.1	wild-type & other
	430162T2	++	1.3	2.5	wild-type & other
	430162T3	++	1.4	3.0	wild-type & other
25	430162T4	+++	1.2	2.1	disrupted
	430162T5	+++	1.0	1.5	disrupted
	430162T6	-	0.9	1.2	wild-type & other

Example 20: Characterization of *Aspergillus oryzae* DEBY1204.3.3 rescued locus pSMO1204

The *Aspergillus oryzae* DEBY1204.3.3 rescued locus pSMO1204 containing 2.0 kb was sequenced according to the procedure described in Example 2. The nucleic acid sequence (SEQ ID NO:34) as shown in Figure 20 had no sequence homology to any published sequences.

Southern analysis and sequencing of a genomic clone was used to confirm that no deletions had taken place when the tagged mutant was generated. A Southern blot of *Aspergillus oryzae* HowB430 genomic DNA digested with various restriction endonucleases (*Bam*HI, *Bgl*II, *Sal*I and *Sph*I) was prepared and analyzed as described in Example 14.

- 5 Probes from both ends of the rescued plasmid were generated by PCR using the primers described below. The primers were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

970052 5'-CTATGATTGGCCGATAGG-3' (SEQ ID NO:35)

970053 5'-CCAGGCTCGCACGCTTTC-3' (SEQ ID NO:36)

- 10 970054 5'-CTTGCAACTAACGGGGTT-3' (SEQ ID NO:37)

970055 5'-TGAGAAAGACCAAGAATG-3' (SEQ ID NO:38)

- Probe 1 was generated from one end of the rescued locus by PCR using primer 970052 and primer 970053. Probe 2 was generated from the other end of the rescued locus by PCR using primer 970054 and primer 970055. The amplification/labeling reaction (50
15 μ l) contained the following components: 10 ng rescued plasmid pSMO1204, 50 pmole each of primer 970052 and 970053 for probe 1 or 50 pmole each of primer 970054 and primer 970055 for probe 2, 1X DIG labeling mix (Boehringer Mannheim, Indianapolis, IN), 1X *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase. The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30
20 cycles each at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5 minutes

- Southern blots of *Aspergillus oryzae* HowB430 genomic DNA digested with various restriction enzymes were prepared and analyzed according to the procedure described in Example 14. The blots were hybridized independently to probe 1 and probe 2. Identical banding patterns of the digests with both probes would suggest that a deletion had not
25 occurred. Conditions of the Southern analysis were as follows: Blots were prehybridized for 1 hour and hybridized overnight at 42°C in Easy Hyb. Blots were washed in 2XSSC, 0.1% SDS twice at room temperature for 15 minutes each, then washed twice at 65°C in 0.1XSSC, 0.1%SDS for 15 minutes each. Detection continued with buffers and reagents from Boehringer Mannheim's DIG Wash Block Buffer System. CDP star (Boehringer
30 Mannheim, Indianapolis, IN) was used to detect the chemiluminescent reaction. Film was exposed for approximately 1 hour.

The Southern blot results showed identical banding patterns with the different digests suggesting a direct insertion of the tagged plasmid. A genomic clone was obtained by probing an *Aspergillus oryzae* HowB430 Ziplox library obtained as described in Example 12 with the probe from the tagged mutant *Aspergillus oryzae* DEBY1204. The Ziplox library
 5 of *Aspergillus oryzae* HowB430 was screened with probe 1. A genomic clone was isolated and sequenced (Example 2) confirming that no deletions had occurred during the tagging event and the tagged plasmid had inserted at an *EcoRI* site.

Example 21: Characterization of *Aspergillus oryzae* mutant HIN603 rescued locus
 10 **pSMO603**

The *Aspergillus oryzae* HIN603 rescued locus pSMO603 containing 1.0 kb was sequenced according to the procedure described in Example 2. The nucleic acid sequence (SEQ ID NO:39) as shown in Figure 21 showed no homology to any published sequences.

A Southern blot of *Aspergillus oryzae* HowB430 genomic DNA digested with the
 15 restriction enzymes *SphI*, *SalI*, and *BamHI* was hybridized to probes made from both ends of the rescued plasmid. Probe 3 was generated by PCR using primer 970858 and primer 970859 shown below synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions. Probe 4 was generated by PCR using primer 970860 and primer 970861. The template for the 50 μ l PCR labeling reaction
 20 was 10 ng of the rescued plasmid pSMOH603. PCR cycles and conditions were as described in Example 20. Southern conditions were as described in Example 20.

970858: 5'-TGTAGTCTGACTAGCATG-3' (SEQ ID NO:40)

970859: 5'-GGATCTTCACCTAGATCC-3' (SEQ ID NO:41)

970860: 5'-CATAGTGTCGACCAAGC-3' (SEQ ID NO:42)

25 970861: 5'-CAATCGAGCTTGCCTATG-3' (SEQ ID NO:43)

Different banding patterns on the Southern suggested that a deletion had taken place where the tagging occurred. When 500 ng of *Aspergillus oryzae* HowB430 genomic DNA was used as a template in a PCR reaction with primers 090858 and 090860, a 3 kb product was amplified suggesting a 2.5 kb deletion had occurred. Southern analysis of genomic
 30 DNA prepared from the tagged *Aspergillus oryzae* HIN603 strain, digested with *HindIII*, and probed with the *NheI* fragment from *Aspergillus oryzae pyrG* suggested that the tagged mutant had not been generated by a REMI event.

Example 22: Construction of *Aspergillus oryzae* HowB432

Aspergillus oryzae HowB432 was generated by transformation of *Aspergillus oryzae* JaL250 with a linear fragment containing the NA2-tpi promoter, a cellulase gene from *Humicola lanuginosa* (CAREZYME™ gene, Novo Nordisk A/S, Bagsværd, Denmark), and the AMG terminator obtained from plasmid pGAG3 (Figure 22).

Aspergillus oryzae JaL250 was constructed from *Aspergillus oryzae* JaL142 (Christensen *et al.*, 1988, *Bio/Technology* 6: 1419-1422) by deleting the neutral protease I gene (*npI*). The *npI* deletion plasmid was constructed by exchanging a 1.1 kb *BalI* fragment coding for the central part of the *npI* gene in plasmid pJaL389 (Figure 23), which contained a 5.5 kb *SacI* genomic fragment encoding the *npI* gene, with a 3.5 kb *HindIII* fragment from pJaL335 (Figure 24) containing the *pyrG* gene flanked by repeats, thereby creating plasmid pJaL399 (Figure 25). *Aspergillus oryzae* JaL142 was transformed with the 7.9 kb *SacI* fragment. Transformants were selected by relief of the uridine requirement on Minimal medium plates. The transformants were analyzed by Southern analysis as described in Example 14 and by IEF protease profile analysis according to standard methods.

Two out of 35 transformants possessed an altered Southern profile compared to the parent strain and displayed no neutral protease I activity by IEF. Furthermore, Southern analysis showed that one of the two transformants had a clean deletion of the *npI* gene and was designated *Aspergillus oryzae* JaL228.

Totally, 2.3×10^7 conidiospores of *Aspergillus oryzae* JaL228 were spread on Minimal medium plates supplemented with 0.1% 5-fluoro-orotic acid (FOA) and 10 mM uridine. Eight FOA resistant colonies were obtained. A Southern blot of *BamHI* digested genomic DNA from the eight colonies probed with a 401 bp *pyrG* repeated region demonstrated that the *pyrG* gene had been excised by recombination at the repeated regions. *Aspergillus oryzae* JaL228 showed two bands of the expected size of 2.7 and 3.1 kb originating from the two copies of the repeated region. If the *pyrG* gene had been lost by recombination between the repeated regions, the 3.1 kb band would have disappeared and only the 2.7 kb would have remained. All 8 FOA resistant colonies showed this pattern of bands. Sequencing of a PCR fragment covering the junctions between the *npI* gene and the copy of the 401 bp repeat remaining in the 8 colonies confirmed that the *pyrG* gene was excised by recombination between the repeats. One of the colonies was designated *Aspergillus oryzae* JaL250.

pGAG3 was constructed by isolating from pDM176 (Figure 26) a *SwaI/PacI* fragment containing the *Humicola lanuginosa* cellulase gene and ligating the fragment into *SwaI/PacI* digested pBANe6. The *SwaI/PacI* fragment from pDM176 and *SwaI/PacI* digested pBANe6 were separated on a 1% agarose gel, and isolated using a QIAquick Gel Extraction Kit (Qiagen Inc., Chatsworth, CA) according to the manufacturer's instructions prior to ligation. The ligation was used to transform *E. coli* DH5 α cells, and the transformants were then screened by extracting plasmid DNA from the transformants using a QIAwell-8 Plasmid Kit according to the manufacturer's instructions, restriction digesting the plasmid DNA to confirm the presence of the correct size fragment, and sequencing the DNA according to the method described in Example 2.

pGAG3 was then digested with *PmeI* and the linear expression cassette was isolated by preparative agarose electrophoresis using TAE buffer. The linear cassette was then used to transform *Aspergillus oryzae* JaL250.

Transformation of *Aspergillus oryzae* JaL250 for *amdS* selection was conducted with protoplasts at a concentration of 2×10^7 protoplasts per ml prepared as described in Example 2. Ten μ g of the linear fragment described above were added to 100 μ l of protoplasts. A volume of 250 μ l of PEG (60% PEG 4000-10 mM CaCl₂-10 mM Tris-HCl pH 8.0) was then added, and the mixture was placed at 37°C for 30 minutes. Three ml of STC medium was added and the mixture was plated on Cove plates supplemented with 10 mM uridine for *amdS* selection. The plates were incubated 7-10 days at 34°C. Transformants were then transferred to plates of the same medium and incubated 3-5 days at 37°C. The transformants were purified by streaking spores and picking isolated colonies using the plates of the same medium without sucrose.

Example 23: *Aspergillus oryzae* transformation with *HpaI* linearized pDSY112 and cellulase expression screening

Aspergillus oryzae HowB432 was transformed with *HpaI* digested pDSY112 and the transformants were recovered using the methods described in Example 5. Totally, 104 recovered transformants were grown in 24 well microtiter plates in 1/4 strength MY25. Samples were taken at 5 and 7 days and assayed for cellulase activity as described below.

Cellulase activity was measured according to the following protocol which is derived from Novo Nordisk method AF 302.1/1-GB which is available from Novo Nordisk A/S,

Bagtsværd, Denmark upon request. A substrate solution containing 2% azo-carboxymethylcellulose was prepared by dissolving the material in 100 mM MOPS pH 7.0 buffer at 80°C for 10 minutes. CAREZYME™ (Novo Nordisk A/S, Bagtsværd, Denmark) was used as a standard. Stock solutions of 2.5 to 25 ECU per ml were prepared to construct a standard curve by diluting accordingly CAREZYME™ in 100 mM MOPS pH 7.0 buffer. Five µl aliquots of the standards and samples (diluted for shakeflasks and fermentations) were pipetted into individual wells of a 96 well plate. A volume of 65 µl of the 2% azo-carboxymethylcellulose solution was pipetted into each of the wells and mixed. The reactions were incubated at 45°C for 30 minutes and then stopped by the addition of 215 µl of stop reagent followed by mixing. The stop reagent was prepared by first suspending 0.2 g of ZnCl₂ in 20 ml of 250 mM MOPS pH 7.0 and adding the suspension to 80 ml of acidified ethanol containing 1.1 ml of concentrated HCl per liter of ethanol. The plate containing the stopped reaction was then centrifuged at 3000 rpm for 10 minutes. A 100 µl aliquot of each supernatant was pipetted into a 96 well plate and the absorbance measured at 600 nm. Using linear regression, the slope, intercept, and correlation coefficient were determined for the standards and samples.

The top ten cellulase producing transformants from the 24 well cultures were spore purified, and regrown in 24 well cultures as above and assayed for cellulase activity. The purified strains were also grown in MY25 in 125 ml shake flasks in MY25 pH 6.5 at 34°C and samples were taken at 3 and 5 days for cellulase assays. *Aspergillus oryzae* HowB432 pDSY112 84-1-1 and *Aspergillus oryzae* HowB432 pDSY112 94-1-1 were also grown in fermentors (2 liters) as described in Example 8. Cellulase activities were measured as described above.

The results from the 24 well and shake flasks cultures are presented in Table 6 where the cellulase yield of *Aspergillus oryzae* HowB432 was normalized to 1.0.

Table 6

Strain		24 well (ECU/ml)	Shake flasks (ECU/ml)	Fermentation results (ECU/ml)
30	HowB432	1.0	1.0	1.0
	C112T50.1.1	2.0	0.9	NA
	C112T84.1.1	2.1	2.0	1.3
	C112T86.1.1	2.3	2.4	NA
	C112T94.1.1	2.5	1.3	1.4
	C112T95.1.1	2.1	1.3	NA
35	C112T100.1.1	2.3	1.6	NA

C112T101.1.1	1.9	1.6	NA
C112T102.1.1	2.0	2.1	NA
C112T103.1.1	1.5	2.0	NA
C112T104.1.1	2.2	2.3	NA

5

A Southern blot of *Aspergillus oryzae* HowB432, *Aspergillus oryzae* DEBY599.3 and the pDSY112 transformants genomic DNAs digested with *Bgl*III was prepared and analyzed as described in Example 14 performed to determine whether pDSY112 had integrated at the homologous locus in the genome using the *Aspergillus oryzae* DEBY599.3 rescued flanking

10 DNA as a probe.

A *Bgl*III band of 2.7 kb from *Aspergillus oryzae* HowB432 hybridized with the probe, while an ~8 kb *Bgl*III band from *Aspergillus oryzae* DEBY599.3 hybridized to the probe. In all of the transformants a wild-type *Bgl*III band of 2.7 kb and a second band corresponding to the transforming DNA hybridized to the probe. Therefore, none of the retransformants

15 had exact gene replacements.

Example 24: *Aspergillus oryzae* transformation with *Nde*I linearized pDSY138 and cellulase expression screening

Aspergillus oryzae HowB432 was transformed with *Nde*I digested pDSY138 using the method described in Example 5. Totally, 240 transformants were recovered which were grown in 24 well microtiter plates in 1/4 strength MY25 as described in Example 8 except samples were taken at days 3 and 5 and assayed for cellulase activity as described in Example 23. The top 20 cellulase producing transformants were spore purified and retested in 24 well microtiter cultures. The top 8 cellulase producing once purified transformants were spore purified a second time and tested in shake flasks in full-strength MY25 as described in Example 8. The top 2 producers were also grown in a 2 liter fermentor as described in Example 8. Cellulase activity was measured as described in Example 23.

The results obtained are shown in Table 7 below where the cellulase yield of *Aspergillus oryzae* HowB432 is normalized to 1.0.

30

Table 7

<u>Strain</u>	<u>ECU/ml</u>	<u>Southern Results</u>
HowB432	1.0	wild-type
C138T21.1.1	1.15	disrupted allele
C138T205.1.1	1.5	wild-type and disrupted alleles

A Southern blot of *Aspergillus oryzae* DEBY932, *Aspergillus oryzae* HowB432, and pDSY138 genomic DNA preparations digested with *Nde*I was prepared and analyzed as described in Example 14 to determine if the pDSY138 DNA had integrated at the homologous locus producing gene replacements in the transformants using the *Aspergillus* 5 *oryzae* DEBY932 rescued flanking DNA as a probe.

The results of the Southern blot demonstrated that an *Nde*I band of approximately 5 kb from *Aspergillus oryzae* HowB432 hybridized to the rescued locus while a band of approximately 10 kb from *Aspergillus oryzae* DEBY932 hybridized to the probe. In Table 7, the column labeled Southern results indicated whether the transformants had a wild-type 10 *Nde*I fragment of the size observed in the parent strain *Aspergillus oryzae* HowB432 or whether the transformants had a band corresponding to the disrupted size observed in *Aspergillus oryzae* DEBY932.

Example 25: *Aspergillus oryzae* transformation with *Ase*I/*Pvu*I *palB* disruption cassette
15 **from pMT1936 and cellulase screening**

Aspergillus oryzae HowB432 was transformed using the same transformation procedure described in Example 5 with a 5.5 kb *Ase*I/*Pvu*I fragment containing the *palB* disruption cassette. The linear fragment for transformation was isolated by digestion of pMT1936 with *Ase*I and *Pvu*I and separation of the fragment on a 1% agarose gel using a 20 QIAquick Gel Extraction Kit according to the manufacturer's instructions. The transformants obtained were then evaluated for growth on Minimal medium plates at pH 6.5 or pH 8.0.

The results showed that 10 of the 312 transformants tested were unable to grow at pH 8.0 indicative of the *palB* minus phenotype. The 10 *palB* minus transformants and 10 of the transformants that were able to grow at pH 8.0 were spore purified and tested in 24 well and 25 shake flask cultures for cellulase production according to the procedures described in Example 23. The results tabulated in Table 8 below demonstrated that *palB* minus strains were better cellulase producers than the *palB* plus strains.

Southern blots of the genomic DNA from an *Aspergillus oryzae palB* minus mutant, an *Aspergillus oryzae palB* plus strain, and *Aspergillus oryzae* HowB432 were performed as 30 described in Example 14 to determine if the *Ase*I/*Pvu*I transforming DNA fragment had integrated as a clean replacement into the *palB* locus.

The results of the Southern blot (Table 8) demonstrated that some of the *palB* minus strains had clean disruptions while others did not.

Table 8

	<u>Strain</u>	<u>PalB phenotype</u>	<u>Shake flasks (ECU/ml)</u>	<u>Southern pattern</u>
5	HowB432	plus	1.0	wild-type
	CpalB5-1	plus	1.0	wild-type and other
	CpalB6-1	plus	1.2	wild-type and other
	CpalB7-1	plus	1.1	wild-type and other
10	CpalB24-1	plus	1.7	wild-type and other
	CpalB28-1	minus	1.6	other
	CpalB34-1	minus	1.6	disruption
	CpalB45-1	minus	1.4	disruption
	CpalB47-1	plus	1.1	wild-type and other
15	CpalB72-1	plus	1.3	wild-type and other
	CpalB76-1	minus	1.6	disruption
	CpalB89-1	minus	1.6	disruption
	CpalB153-1	minus	1.1	disruption
	CpalB161-1	plus	1.2	wild-type and other
20	CpalB163-1	minus	1.9	other
	CpalB185-1	minus	1.8	other
	CpalB190-1	minus	1.4	disruption

**Example 26: *Aspergillus oryzae* transformation with *AsnI/PvuI* manganese superoxide
25 dismutase disruption cassette and cellulase screening**

Aspergillus oryzae HowB432 was transformed with a 5.8 kb *AsnI/PvuI* fragment containing the manganese superoxide dismutase disruption cassette according to the same procedure described in Example 5.

Twenty transformants were obtained and tested for sensitivity to paraquat as described
30 in Example 17. Seven of the 20 transformants were paraquat sensitive indicative of the manganese superoxide dismutase minus phenotype although they are sensitive to different levels of paraquat as indicated in Table 9 below. Those indicated as + + + for paraquat sensitivity are sensitive to as low as 2 mM paraquat, while those labeled - and + + are not sensitive to paraquat and sensitive to intermediate levels of paraquat, respectively. All of the
35 transformants were spore purified and tested in 24 well cultures for cellulase production as described in Example 23. The strains were also tested in 125 ml shake flasks cultures as described in Example 23. The results are shown in Table 9 below. The strains that are

paraquat sensitive produce on average more CAREZYME™ than those strains that are not paraquat sensitive.

Southern blots of the transformants and *Aspergillus oryzae* HowB432 were prepared and analyzed as described in Example 14 to determine if the *AsnI/PvuI* manganese superoxide dismutase disruption cassette had integrated to give a clean replacement into the manganese superoxide dismutase disruption cassette locus.

The results of the Southern blot shown in Table 9 below indicate that the strains sensitive to 2 mM paraquat have only the disrupted locus of manganese superoxide dismutase, while those sensitive to intermediate levels of paraquat have both the wild-type locus and the disrupted cassette. The intermediate sensitivity to paraquat may be explained by the fact that manganese superoxide dismutase is a homodimer, and those cells expressing the wild-type and truncated manganese superoxide dismutase coded for by the disruption cassette would be producing heterodimers that are probably not functional.

Table 9

	<u>Strain</u>	<u>Paraquat sensitivity</u>	<u>Shake flasks</u>	
			<u>(Relative LU/ml)</u>	<u>Southern Results</u>
	HowB432	-	1.00	wild-type
	432162T3	+++	1.26	disrupted
	432162T7	++	1.24	wild-type & other
	432162T8	-	1.21	wild-type & other
	432162T9	-	0.61	wild-type & other
	432162T10	++	1.14	wild-type & other
	432162T11	++	1.04	wild-type & other
	432162T12	++	1.12	wild-type & other
	432162T15	-	0.44	wild-type & other
	432162T16	++	1.10	wild-type & other
	432162T17	+++	1.53	-
	432162T18	-	1.12	-
	432162T19	+++	1.44	-

Example 27: Construction of glucose transporter gene overexpression plasmids pHB218 and pDSY153 and stop control plasmids pDSY152 and pDSY155

Plasmids to overexpress the glucose transporter rescued locus from *Aspergillus oryzae* DEBY599.3 were constructed to determine if overexpression of the glucose transporter would lead to an increase in the yields of *Humicola lanuginosa* lipase and cellulase. The glucose transporter open reading frame was PCR amplified to place *SwaI* and *PacI* sites at the 5' and 3' end of the ORF, respectively. The following primers synthesized with an Applied

Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions were used in combination with 0.2 μ g of pDSY112 in the amplification:

961176: 5'-ATTTAAATGGTCCTCGGTGGATCAAGC-3' (SEQ ID NO:44)

961177: 5'-TTAATTAATTAGTCCTGTCTGCGCTGGT-3' (SEQ ID NO:45)

5 The conditions and parameters used for the amplification are described in Example 2. Ten μ l of the PCR reaction was electrophoresed on an agarose gel, and a 1.5 kb product was obtained as expected. The PCR product was cloned using a pPCR-Script™ Kit (Stratagene, La Jolla, CA) according to the manufacturer's protocols. The ligation reaction was used to transform *E. coli* DH5 α cells, and plasmid DNA was isolated from several of the transformants using the QIAWell-8 Plasmid Kit. The plasmids were digested with *NotI* and *EcoRI* to determine which clones had the 1.5 kb insert. Six of the 11 clones analyzed had the correct size insert as determined by electrophoreses on an agarose gel. One of the clones, pDSY119, was digested with *PacI* and *SwaI*, and the digest was run on an agarose gel. The 1.5 kb *SwaI/PacI* band was excised from the gel, and DNA was purified from the gel slice using the QIAQuick Gel Extraction Kit. The 1.5 kb fragment was ligated with *SwaI/PacI* cut pBANe13 (Figure 3) using standard conditions (Sambrook *et al.*, 1989, *supra*). The ligation was used to transform *E. coli* DH5 α cells, and plasmid DNA was isolated from several of the transformants. The plasmids were digested with *SwaI/PacI* to determine which clones had the expected 1.5 kb insert. The final plasmid was designated pHB218 (Figure 20 27).

As a control for the overexpression experiments, a derivative of pHB218 in which a stop codon was inserted at amino acid 9 in the glucose transporter open reading frame was made using site-directed mutagenesis. A MORPH™ Site-Specific Plasmid DNA Mutagenesis kit from 5 Prime→ 3 Prime was used for the mutagenesis, and protocols provided with the kit were followed. The reaction contained pHB218 as template, and the mutagenic primer used was:

970545: 5'-CGGTGGATCAAGCGGTTAATTAATCACTCCGTACCTGAT-3' (SEQ ID NO:46)

Several *E. coli* colonies were obtained after following the protocols, and plasmid DNA was isolated from the colonies using the QIA-Well8 plasmid kit. The plasmids were digested with *PacI* since the mutagenic primer introduced a *PacI* site which served as a marker for the mutagenesis. Two of the plasmids with the extra *PacI* site indicative of a successful mutagenesis were sequenced as described in Example 2 to confirm the presence

of the stop codon at amino acid 9 in the ORF. The pHB218 derivative with the stop codon at amino acid 9 was designated pDSY152.

Versions of pHB218 and pDSY152 in which the selectable marker was the *bar* gene were constructed for transformation of strains which are *pyrG* plus. The *SwaI/PacI* inserts from pHB218 and pDSY152 were isolated by restriction digestion, electrophoresed on an agarose gel, and purified using QIAQuick Gel Extraction Kit. The inserts were ligated into pSE39 (Figure 28) and digested with *SwaI/PacI*. The ligation reaction was used to transform *E. coli* DH5 α , and plasmid DNA was isolated from the colonies as described above. The plasmids were digested with *SwaI/PacI* to determine which clones contained the expected 1.5 kb insert. The plasmids were sequenced as described in Example 2 to confirm the presence or absence of the stop codon at amino acid 9 in pDSY155 and pDSY153 (Figure 29), respectively. The only difference between pDSY155 and pDSY153 was the stop codon at amino acid 9 of the glucose transporter ORF in pDSY155.

Example 28: Transformation of *Aspergillus oryzae* HowB430 and *Aspergillus oryzae* HowB432 with pHB218 and pDSY152 and lipase and cellulase screening, respectively.

Aspergillus oryzae HowB430 was transformed with pHB218 or pDSY152, and the transformants were recovered using the methods described in Example 5. One hundred and twenty transformants each with pHB218 and pDSY152 were recovered, grown in 24-well microtiter plates in 1/100 strength MY25 and assayed for lipase activity after 3 and 5 days as described in Example 8. The assay results showed that there was a slight shift towards higher lipase production in the pHB218 transformants versus the pDSY152 transformants supporting the idea that overexpression of the glucose transporter has a positive effect on lipase expression.

Aspergillus oryzae HowB432 was transformed with pHB218 and pDSY152, and the transformants were recovered using the methods described in Example 5. One hundred transformants each with pHB218 and pDSY152 were recovered, grown in 24-well microtiter plates in 1/4 strength MY25 and assayed for cellulase activity after 3 and 5 days as described in Example 23. The assay results showed that there was a shift towards higher cellulase production in the pHB218 transformants versus the pDSY152 transformants indicating that overexpression of the glucose transporter had a positive effect on cellulase expression.

Example 29: Transformation of *Aspergillus oryzae* DEBY10.3 with pDSY153 and pDSY155 and lipase screening.

Aspergillus oryzae DEBY10.3 was transformed with pDSY153 and pDSY155, and the transformants were recovered using the methods described in Example 5. Two hundred sixteen and 144 transformants with pDSY153 and pDSY155, respectively, were recovered, grown in 24-well microtiter plates in 1/100 strength MY25, and assayed for lipase activity on days 4 and 6 as described in Example 8. There was shift towards higher lipase production in the pDSY153 transformants when compared to the pDSY155 transformants indicating that overexpression of the glucose transporter led to an increase in lipase production and also suggesting that the *palB* minus effect and the glucose transporter overexpression were additive.

Example 30: Identification of tagged event in *Aspergillus oryzae* HowL795

Genomic DNA was prepared from *Aspergillus oryzae* HowL795 according to Example 9. One μ g of DNA was digested with either *Sna*BI or *Nsi*I. Both enzymes cleave within the *pyrG* gene contained on the tagging construct. The DNA was then diluted to 4 ng/ μ l and recircularized with T4 Ligase at 22°C for 18 hours. Inverse PCR was then performed using approximately 500 ng of recircularized DNA using the primers shown below which were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions. Both were located downstream of the *Nsi*I and *Sna*BI sites.

Primer x: 5'-GCACTCGAATGACTACT-3' (SEQ ID NO:47)

Primer y: 5'-CGCATCATACTTGCGACA-3' (SEQ ID NO:48)

The inverse PCR amplification reaction contained the following components: 500 ng of recircularized DNA, 150 pmoles of primer x, 150 pmoles of primer y, 1 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase. The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 5 minutes followed by 30 cycles each at 95°C for 1 minute, 55°C for 1 minute and 72°C for 2 minutes. The PCR product was isolated by electrophoresis on a 1% agarose gel.

PCR of the religated *Sna*BI DNA amplified a 4 kb fragment whereas PCR of the religated *Nsi*I DNA amplified a 2 kb fragment. PCR confirmed that the smaller *Nsi*I fragment was contained within the larger *Sna*BI fragment.

DNA sequence analysis was performed according to the procedure described in Example 2 using primer A. The analysis identified that the insertion of pSO122 had occurred in the 3' non-translated region of the *amdS* gene contained within plasmid pBANe8.

5 Example 31: Construction of *Aspergillus oryzae* MStr107

Aspergillus oryzae MStr107 was constructed to contain extra copies of one of the native alpha-amylase (TAKA) genes (FUNGAMYL™ gene, Novo Nordisk A/S, Bagsværd, Denmark), by transforming *Aspergillus oryzae* HowB101 with a DNA fragment from pMStr15. pMStr15 was constructed from pCaHj505 and pTAKA17 as described below.

10 Standard methods were employed (Sambrook *et al.*, 1989, *supra*) except where noted.

pCaHj505 (Figure 30) was constructed to contain the *Aspergillus oryzae* NA-14 alpha-amylase (TAKA) promoter, the *Aspergillus niger* glucoamylase (AMG) terminator, and the *Aspergillus nidulans amdS* gene from the following fragments:

- a) The vector pToC65 (WO 91/17243) digested with *EcoRI* and *XbaI*.
- 15 b) A 2.7 kb *XbaI* fragment from *Aspergillus nidulans* carrying the *amdS* gene (Corrick *et al.*, 1987, *Gene* 53: 63-71). The *amdS* gene was used as a selective marker in fungal transformations. The *amdS* gene was modified so that the *BamHI* site normally present in the gene was destroyed. This was done by introducing a silent point mutation using the primer: AGAAATCGGGTATCCTTTCAG (SEQ ID NO:49).
- 20 c) A 0.6 kb *EcoRI-BamHI* fragment carrying the *Aspergillus oryzae* NA-14 alpha-amylase promoter.
- d) A 675 bp *XbaI* fragment carrying the *Aspergillus niger* glucoamylase transcription terminator. The fragment was isolated from the plasmid pICAMG/Term (EP 238 023).

The *BamHI* site of fragment c was connected to the *XbaI* site in front of the transcription terminator on fragment d via the pIC19R linker (*BamHI* to *XbaI*) (Boehringer Mannheim, Indianapolis, IN).

pMStr15 (Figure 31) was constructed to contain the *Aspergillus oryzae* NA-14 alpha-amylase promoter, gene and terminator and the *Aspergillus nidulans amdS* gene. The alpha-amylase gene with promoter and terminator was excised from pTAKA17 (European patent 30 0238 023) as a 2.9 kb *EcoRI-HindIII* fragment and cloned adjacent to the *amdS* gene in the vector pCaHj505 by replacing the *EcoRI-XbaI* promoter/terminator fragment in pCaHj505.

To facilitate cloning, the recessed 3' termini generated by *Hind*III and *Xba*I digestion were filled in.

A single linear DNA fragment containing both the alpha-amylase gene and the *amdS* gene was obtained by digesting pMStr15 with *Not*I, resolving the vector and insert sequences using agarose gel electrophoresis, excising the appropriate DNA band from the gel, and purifying the DNA from the agarose using GenElute™ Agarose Spin Columns according to the manufacturer's directions (Supelco, Bellefonte, PA). This 5.6 kb *Not*I fragment was used to transform *Aspergillus oryzae* HowB101 to construct *Aspergillus oryzae* MStr107, using the transformation protocol and selective medium described in Example 2. Transformants were propagated from single colonies twice in succession on COVE medium with 0.1% Triton X100 before performing additional screens.

Aspergillus oryzae MStr107 was selected from among the transformants based on its ability to produce more alpha-amylase than *Aspergillus oryzae* HowB101. The ability of the transformants to produce alpha-amylase was determined by culturing them in 10 ml of YPM medium for 4 days at 30°C with shaking and resolving 5 µl of the culture medium by SDS-PAGE according to standard methods. The strain producing the most alpha-amylase under these conditions was selected as *Aspergillus oryzae* MStr107, and was compared in a 3 liter fermentation culture to *Aspergillus oryzae* HowB101. The medium was composed of maltose syrup, yeast extract, KH₂PO₄, K₂SO₄, (NH₄)₂SO₄, citric acid, MgSO₄, trace metals and uridine. Under these conditions, *Aspergillus oryzae* MStr107 produced 360% of *Aspergillus oryzae* HowB101.

Example 32: *Aspergillus oryzae* Mstr107 transformation with linearized pDSY82

Protoplasts of *Aspergillus oryzae* Mstr107 were prepared as described in Example 2. A 5-15 µl aliquot of pDSY82 (6 µg) linearized with 1.25 U of *Xba*I was added to 0.1 ml of the protoplasts at a concentration of 2 x 10⁷ protoplasts per ml in a 14 ml Falcon polypropylene tube followed by 250 µl of 60% PEG 4000-10 mM CaCl₂-10 mM Tris-HCl pH 7, gently mixed, and incubated at 37°C for 30 minutes. Three ml of SPTC were then added and the suspension was gently mixed. The suspension was mixed with 12 ml of molten overlay agar (1X COVE salts, 1% NZ amine, 0.8 M sucrose, 0.6% Noble agar) or 3 ml of STC medium and the suspension was poured onto a Minimal medium plate. The plates were incubated at 37°C for 3-5 days.

The transformation frequency of *Aspergillus oryzae* MStr107 with pDSY82 and *Xba*I was approximately 200 transformants/ μ g of DNA. A library of approximately 30,000 transformants was obtained. Spores from 70 pools with approximately 400 transformants in each pool were collected and stored in a 20% glycerol, 0.1% Tween 80 at -80°C. The pools and transformants from these libraries were designated with the letter "x".

Example 33: Characterization of integration events in "REMI" *Aspergillus oryzae* MStr107 transformants

Transformants of *Aspergillus oryzae* MStr107 with pDSY82 and *Xba*I (library "x") were analyzed as described in Example 6. Genomic DNA was isolated from 40 transformants, 20 from one pool (x15) and 20 from 20 various pools. DNA samples were cut with *Hind*III, resolved, blotted and probed with radiolabeled pDSY82. Thirty-three of 40 displayed apparently novel band patterns, suggesting that plasmid integrations were distributed to different sites in the genome. For 19 of the 40 transformants the band patterns suggested that only one copy of pDSY82 integrated in the genome, while more than one copy was observed in the remaining 21 transformants. DNA from the 20 transformants taken from various pools was also cut with *Xba*I, resolved, blotted and probed with radiolabeled pDSY82 as described in Example 6. A single, plasmid-sized band was observed indicating REMI had occurred at an *Xba*I site in 9 of the transformants.

Example 34: FUNGAMYL™ expression screening

The *Aspergillus oryzae* MStr107 tagged mutant library "x" pools described in Example 32 were assayed for FUNGAMYL™ expression.

For 96-well plate screens, MTBCDYU medium was used. For 24-well plate methods, 4xMTBCDYU medium was used.

Primary 96-well plate screens involved the dilution of spores from distinct pools into MTBCDYU so that one spore on average was inoculated per well when 100 μ l of medium was dispensed into the wells. After inoculation, the 96-well plates were grown for 3-4 days at 34°C under static conditions. Cultures were then assayed for FUNGAMYL™ activity as described below. Mutants of interest were isolated twice on YPG or Cove plates, and single colonies transferred to Cove agar slants. Spores from Cove slants were inoculated into 24-well plates containing 4xMTBCDYU with approximately 10^3 spores per well and grown

under static conditions for 4 days at 34°C. Cultures were then assayed for FUNGAMYL™ activity as described below.

The FUNGAMYL™ assay substrate (4-nitrophenyl-alpha-D-maltoheptasid-4,6-O-ethyliden, EPS) was prepared as a 1/2 strength solution relative to the instructions given by the manufacturer (Boehringer Mannheim, Indianapolis, IN). The substrate was prepared in HEPES pH 7.0 buffer. A FUNGAMYL™ standard (FUNGAMYL™, Novo Nordisk A/S, Bagsværd, Denmark) was prepared to contain 2 FAU/ml in HEPES pH 7.0 buffer. The standard was stored at -20°C until use. FUNGAMYL™ stock was diluted appropriately to obtain a standard series ranging from 0.02 to 0.2 FAU/ml just before use. Broth samples were diluted in HEPES buffer and 25 µl aliquots were dispensed to wells in 96-well plates followed by 180 µl of diluted substrate. Using a plate reader, the absorbance at 405 nm was recorded as the difference of two readings taken at approximately 1 minute intervals. FUNGAMYL™ units/ml (FAU/ml) were calculated relative to the FUNGAMYL™ standard solutions.

The results of the 96-well screen followed by the 24-well screen identified for further evaluation 51 transformants from the pDSY82 and *Xba*I transformations. These identified transformants produced higher levels of FUNGAMYL™ than the control strain *Aspergillus oryzae* MStr107.

Example 35: Shake flask and fermentation evaluation

The highest FUNGAMYL™-producing DNA-tagged mutants described in Example 34 were evaluated in shake flasks and fermentors.

Shake flask evaluations were performed by inoculating half the spore content of a COVE slant suspended in a suitable volume of sterile water containing 0.02% TWEEN-80 into 100 ml of G1-gly medium at pH 7.0 in a 500 ml shake flask. The G1-gly shake flasks were incubated at 34°C for 1 day at 270 rpm. Next, 5 ml of the G1-gly cultures were inoculated into 100 ml of 1/5MDU2BP at pH 6.5 in 500 ml shake flasks. Samples were taken at day 3, and FUNGAMYL™ activity was measured as described in Example 34.

The DNA-tagged mutant X70-25 and 257D11 were grown in a 3 liter lab fermentor containing a medium composed of Nutriose, yeast extract, MgSO₄, KH₂PO₄, citric acid, K₂SO₄, (NH₄)₂SO₄ and trace metals solution. The fermentation was performed at a temperature of 34°C, a pH of 7, and the agitation was maintained between 1000-1200 rpm

for 5 days. FUNGAMYL™ activity was measured by partial degradation of EPS to G₂ and G₃ derivatives (G=glucose). The G₂ and G₃ derivatives were then degraded to glucose and yellowish colored p-nitrophenolate anion by the addition of a surplus of alpha-glucosidase. The analytical output was determined as the change in absorbance at 405 nm per unit time
 5 (3 minutes) at 37°C and pH 7.1 after a preincubation for 2.5 minutes. FUNGAMYL™ was used as standard.

The results obtained are shown in Table 10 below where the FUNGAMYL™ yield of *Aspergillus oryzae* MStr107 as a control is normalized to 1.0.

Table 10 - FUNGAMYL™ Expression by DNA Tagged Mutants

Strain Description	Pool	# Screened	24-well	Shake Flask	Fermentation
			in 96-well Plates (FAU/ml)	Plate Results (FAU/ml)	Results (FAU/ml)
HowB101	NA	NA	0.4	0.4	0.3
MStr107	NA	NA	1.0	1.0	1.0
X70-25	x70	580	1.4	1.3	1.2
257D11	x6	480	1.5	1.4	1.0
X70-42	x70	580	1.3	1.3	ND
263A3	x6	480	1.4	1.6	ND
X69-246	x69	350	1.6	1.4	ND
X59-122	x59	580	1.3	1.3	ND
X49-233	x49	460	1.4	1.4	ND

As shown in Table 10, the mutants produced approximately 30-60% more FUNGAMYL™ than the control strain *Aspergillus oryzae* MStr107 when grown in 24-well plates and when grown in shake flasks. The mutant, *Aspergillus oryzae* X70-25 produced approximately 20% more FUNGAMYL™ than the control strain *Aspergillus oryzae* MStr107
 30 when grown in fermentors.

Example 36: Screening for morphological mutants

The 5 "e" pools and 5 "b" pools described in Example 5 were screened for altered morphology by plating on CM-1 agar and incubating at 34°C for 4 days.

35 Twenty-four colonies having altered plate morphology and covering the morphological variation within the pool were transferred to fresh CM-1 plates and incubated 5 days at 34°C for single colony isolation. Each morphology (in most cases 1) on a plate, was transferred

from a single colony to the center of a CM-1 plate and a PDA plate, and incubated 6-8 days at 34°C before the morphology was evaluated, *i.e.*, the diameter and the appearance. A total of 218 morphological mutants was transferred to COVE plates and incubated at 34°C for 1-2 week to generate spores.

5

Example 37: Evaluation of morphological mutants

The morphological mutants isolated in Example 36 were evaluated in 24-well plates for lipase production according to the procedure described in Example 7. The highest yielding mutants were compared with respect to plate morphology on CM-1 agar, and 23
10 mutants covering the observed morphological variation were further tested in shake flasks containing CD medium to evaluate lipase production.

Approximately 0.25 ml of spore suspension from a CM-1 plate was inoculated into 25 ml of G1-gly medium in a 125 ml PP flask and incubated at 34°C for 24 hours. Then 0.5 ml of the 24 hour seed flask was transferred to 50 ml of CD medium supplemented with
15 1.0 µl of FUNGAMYL™ 800L (Novo Nordisk A/S, Bagsværd, Denmark) in a 125 ml PP flask, and incubated at 34°C, 200 rpm. The culture was sampled after 2 and 3 days and assayed for lipase activity as described in Example 7.

The isolated mutants were also tested in the following manner in oxygen limited media. *Aspergillus oryzae* HowL536.3 was run as a control since the strain possessed the
20 wild type morphology and did not require uridine for growth. Approximately 250 µl of spore suspension was inoculated into a 125 ml shake flask containing 25 ml OL-1 medium and incubated at 34°C, 200 rpm until residual glucose was < < 1 g/l measured using DIASTIX™ (Bayer, Elkhart, IN). Then 75 ml OL-6 medium was added to each flask and further incubated at 34°C, 200 rpm for approximately 25 hours until residual glucose in the
25 *Aspergillus oryzae* HowL536.3 flask was approximately 5 g/l. At that time, all the flasks were assayed for residual glucose, and the flasks with significantly lower glucose (0-2 g/l) were considered positive. The majority of the flasks averaged around 5-10 g/l.

Twenty mutants converting the glucose faster than average were considered likely to be easier to aerate and were further tested in shake flasks containing CD medium as
30 described above to evaluate lipase expression.

Based on these tests, 14 mutants were identified and further evaluated in lab fermentors according to the procedure described in Example 8. The morphological mutants listed below in Table 11 were identified.

Table 11: Morphological mutants

5	<u>Strain</u>	<u>Construction</u>	<u>Pool</u>	<u>Description</u>
	P2-7.1	pDSY82+BamHI	b2	colonial, easy to aerate, 50% yield increase in fermentors
	P3-2.1	pDSY82+BamHI	b3	flat, yield 40% yield increase in fermentors
	P4-8.1	pDSY82+BamHI	b5	easy to aerate, 30% yield increase in fermentors
10	P5-7.1	pDSY82+BamHI	b6	easy to aerate, 60% yield increase in fermentors
	P7-14.1	pDSY81+EcoRI	e2	colonial, easy to aerate, 50% yield increase in fermentors
	P8-10.1	pDSY81+EcoRI	e3	easy to aerate, yield increased 50% in fermentors

15 **Example 38: Rescue of plasmid DNA and flanking DNA from morphological mutants**

The plasmid DNA and genomic flanking loci were isolated from mutants *Aspergillus oryzae* P4-8.1 and P7-14.1 using the procedure described in Example 9 except for the restriction endonuclease(s) used. Transformant *E. coli* HB101 p4-8.1 contained a *Bgl*II rescued locus from mutant *Aspergillus oryzae* P4-8.1. Transformant *E. coli* HB101 p7-14.1
20 contained a *Nar*I rescued locus from mutant *Aspergillus oryzae* P7-14.1.

The plasmid DNA and genomic flanking loci were isolated from mutants *Aspergillus oryzae* DEBY7-17.2, DEBY3-2.1, DEBY5-7.1, and DEBY8-10.1. The rescued plasmids were generated as previously described in Example 9 with the exception that rescues pSMO717, pSMO321, pHowB571, and pSMO810 were isolated from transformed *E. coli*
25 DH5 α cells.

Transformant *E. coli* DH5 α pSMO717 contained the *Bgl*II rescued locus from mutant *Aspergillus oryzae* DEBY7-17.2. Transformant *E. coli* DH5 α pSMO321 contained the *Bgl*II rescued locus from mutant *Aspergillus oryzae* DEBY3-2.1. Transformant *E. coli* DH5 α pHowB571 contained the *Nde*I rescued locus from mutant *Aspergillus oryzae* DEBY5-7.1.
30 Transformant *E. coli* DH5 α pSMO810 contained the *Nde*I rescued locus from mutant *Aspergillus oryzae* DEBY8-10.1.

Example 39: Characterization of morphological mutant *Aspergillus oryzae* P4-8.1 rescued locus p4-8.1

The *Aspergillus oryzae* P4-8.1 rescued locus p4-8.1 containing 915 and 665 bp regions on either side of the integration event was sequenced according to the procedure described in Example 2. The nucleic acid sequence (SEQ ID NO:50) and the deduced amino acid sequence (SEQ ID NO:51) are shown in Figure 32. The nucleic acid sequence suggested that the integration event occurred within an open reading frame for a homologue of the *Saccharomyces cerevisiae* YHM4 Heat Shock protein gene. The deduced amino acid sequence (SEQ ID NO:51) showed 40.2% identity to the *Saccharomyces cerevisiae* YHM4 Heat Shock protein (SEQ ID NO:52) and 41.8% identity to a *Schizosaccharomyces pombe* Heat Shock Protein 70 (SEQ ID NO:53).

Example 40: *Aspergillus oryzae* transformation with *Bgl*III linearized p4-8.1 and morphology screening

To verify the link between the observed plate morphology for *Aspergillus oryzae* P4-8.1 and the rescued genomic locus, *Aspergillus oryzae* HowB430 was transformed with the *Bgl*III linearized rescued locus of *Aspergillus oryzae* P4-8.1, p4-8.1, using the procedure described in Example 5.

Sixty-six transformants were obtained, transferred to CM-1 agar, and incubated at 34°C for 3-4 days to evaluate the morphology. Sixteen transformants with the correct plate morphology were transferred to fresh CM-1 plates as center colonies, and 12 transformants maintaining the plate morphology after 4 days at 34°C were analyzed by Southern blot analysis with a PCR amplified 300 bp fragment of the rescued locus as a probe. The fragment was PCR amplified using the primers below synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions.

HSP-1: 5'-TACGGTTGACAGTGGAGC-3' (SEQ ID NO:54)

HSP-3r: 5'-CACTGACTTCTCCGATGC-3' (SEQ ID NO:55)

The amplification reaction (50 µl) contained the following components: 0.2 ng of p4-8.1, 50 pmol of primer HSP-1, 50 pmol of primer HSP-3r, 0.25 mM each of dATP, dCTP, dGTP, and dTTP, 1 x *Taq* polymerase buffer, and 2.5 U of *Taq* polymerase. The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 1.5

minutes; and 1 cycle at 72°C for 5 minutes. The PCR product was isolated by electrophoresis on a 1% agarose gel.

Samples of the genomic DNA were obtained from each of the 12 transformants obtained according to the method described in Example 9. The genomic DNAs were
5 digested with *Bgl*III and submitted to Southern analysis according to the procedure described in Example 14.

All 12 transformants were affected at the rescued locus, suggesting a connection between this locus and the observed plate morphology.

10 **Example 41: Characterization of morphological mutant *Aspergillus oryzae* P7-14.1 rescued locus p7-14.1**

The *Aspergillus oryzae* P7-14.1 rescued locus p7-14.1 containing 1040 and 520 bp regions on either side of the integration event was sequenced according to the procedure described in Example 2. The nucleic acid sequence (SEQ ID NO:56) and the deduced amino
15 acid sequence (SEQ ID NO:57) are shown in Figure 33. The nucleic acid sequence suggested that the integration event occurred within an open reading frame for a homologue of the *Aspergillus nidulans* chitin synthase B (*chsB*) gene and the *Aspergillus fumigatus* chitin synthase G (*chsG*) gene. Identities of 94% and 80% were found when the deduced amino acid sequences of the two sides of the rescued locus (SEQ ID NO:57), the *chsB* gene (SEQ
20 ID NO:58), and the *chsG* gene (SEQ ID NO:59) were compared.

Disruption of the *chsB* gene in *Aspergillus nidulans* is known to change the morphology significantly (Yanai *et al.*, 1994, *Biosci. Biotech. Biochem.* 58: 1828-1835), and in *Aspergillus fumigatus* disruption of the *chsG* gene is known to cause colonial morphology (Mellado *et al.*, 1996, *Molecular Microbiology* 20: 667-679), which is the observed
25 phenotype of *Aspergillus oryzae* P7-14.1.

Example 42: *Aspergillus oryzae* transformation with a linear *chs* fragment and morphology screening

A 1.9 kb DNA fragment was generated by PCR using as the template *Aspergillus*
30 *oryzae* HowB430 genomic DNA prepared as described in Example 6. Primer A, 5'-CACCAAGTCAGAGCGTC-3' (SEQ ID NO:60), was derived from the rescued *chs* *Aspergillus oryzae* homolog. Primer 5, 5'-GGICCITTYGAYGAYCCICA-3' (SEQ ID

NO:61), was degenerate based on the consensus sequence of the *Aspergillus fumigatus chsG* and *Aspergillus nidulans chsB* genes. The amplification reaction (50 μ l) contained the following components: 10 ng of pHB220, 48.4 pmol of each primer, 1 mM each of dATP, dCTP, dGTP, and dTTP, and the Advantage-GC™ Tth Polymerase Mix (Clontech, Palo Alto, CA). The reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 3 minutes; and 1 cycle at 72°C for 5 minutes. The PCR product was isolated by electrophoresis on a 1% agarose gel.

The DNA fragment was cloned into the PCR-Blunt Cloning Vector (Invitrogen, San Diego, CA). A *HindIII* site in the multicloning site was destroyed by filling in with the Klenow fragment of DNA Polymerase I. A 2 kb *HindIII-EcoRI* fragment containing the Basta gene conferring resistance to Bialaphos was obtained from pMT1612 (Figure 34) and inserted into the *chs HindIII* site located approximately 0.7 kb within the *chs* fragment. The resultant plasmid was labelled pHB220.

Using pHB220 as template, a 4 kb PCR fragment was generated using primer A and 5'-GGGCCGTTTGACAATCCGCAT-3' (SEQ ID NO:62). The amplification reaction was performed as described above except the reaction was incubated in an Ericomp Thermal Cycler programmed as follows: One cycle at 95°C for 3 minutes; 30 cycles each at 95°C for 1 minute, 58°C for 1 minute, and 72°C for 6 minutes; and 1 cycle at 72°C for 5 minutes. The PCR product was isolated by electrophoresis on a 1% agarose gel.

The PCR fragment was then used directly to transform protoplasts prepared from *Aspergillus oryzae* HowL795 according to the procedure described in Example 5. Of 100 transformants, three transformants appeared to have a "colonial" plate morphology on Minimal medium plates and PDA plates.

Southern analysis was performed on the three transformants and *Aspergillus oryzae* HowB430 as a control using the 2 kb DIG-labelled *chs* fragment as probe. Genomic DNA was prepared from the three strains and control strain as described in Example 9. Samples of the genomic DNA from each of the 3 transformants digested with *HindIII* was submitted to Southern analysis according to the procedure described in Example 14.

The Southern analysis showed that each of the three transformants had undergone a gene replacement substituting the *chs/basta* construct with the wild-type *chs* gene. The

results confirmed that the colonial morphology observed in the *chs* tagged strain *Aspergillus oryzae* P7-14.1 was associated with a mutation of the *chs* gene.

An apparent effect of the *chs* gene on colony morphology was observed in shake flask cultures containing MY25 medium performed as described in Example 8. The pellet mass
5 of the colonies in the broth appeared less dense in the *chs* mutants of *Aspergillus oryzae* HowL795 compared to *Aspergillus oryzae* HowL795.

Fermentations were also performed as described in Example 8 on two derivatives of *Aspergillus oryzae* HowL795 containing gene disruptions of the *chs* gene. Lipase yields in both strains were approximately 21 % greater than *Aspergillus oryzae* HowL795. The kinetics
10 of enzyme production appeared to be increased in the *chs* mutants in the later stages of fermentation suggesting that these strains exhibited a more optimal tank morphology.

Example 43: Characterization of morphological mutant *Aspergillus oryzae* DEBY7-17.2 rescued locus pSMO717

15 The *Aspergillus oryzae* DEBY7-17.2 rescued locus pSMO717 containing 400 bp was sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ ID NO:63) and the deduced amino acid sequence (SEQ ID NO:64) are shown in Figure 35. The deduced amino acid sequence (SEQ ID NO:64) showed 44% identity to the deduced amino acid sequence of an ORF of *Aspergillus nidulans* (AC000133) (SEQ ID NO:65).

20

Example 44: Characterization of morphological mutant *Aspergillus oryzae* DEBY3-2.1 rescued locus pSMO321

The *Aspergillus oryzae* DEBY3-2.1 rescued locus pSMO321 containing 1.0 kb was sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ
25 ID NO:66) shown in Figure 36 showed no homology to any published sequences.

Probes from either end of the rescued plasmid pSMO321 were generated by PCR. Probe 5 was generated with primers 970850 and 970851 shown below. Probe 6 was generated with primers 970852 and primer 970853 shown below. The primers were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the
30 manufacturer's instructions. The template for the 50 μ l PCR labeling reaction was 10 ng of the rescued plasmid pSMO321. PCR cycles and conditions were as described in Example 20.

970850: 5'-GTTCTATTGAGATACGCG-3' (SEQ ID NO:67)

970851: 5'-ACAAGCCGACCGGTTTTG-3' (SEQ ID NO:68)

970852: 5'-CGATAAGGACTCCAAGAG-3' (SEQ ID NO:69)

970853: 5'-GTCGCGCATAATATGAAG-3' (SEQ ID NO:70)

5 Southern blots of *Aspergillus oryzae* HowB430 genomic DNA digested with *Sph*I, *Sal*I and *Bam*HI were prepared and analyzed according to the method described in Example 14. The blots were hybridized independently to probes 5 and 6 made from the ends of the rescued plasmid.

10 Analysis of the Southern blots suggested no deletions had occurred. When PCR was performed using 500 ng of genomic DNA from *Aspergillus oryzae* HowB430 with primers 090850 and 090852, a 500 bp product was amplified as predicted verifying that no deletions had taken place.

15 Genomic DNA was prepared from the tagged mutant strain *Aspergillus oryzae* DEBY3-2.1 as described in Example 9, digested with the restriction enzyme used for REMI (*Bam*HI), blotted and probed with the *Nhe*I fragment from *Aspergillus oryzae* *pyrG*. Southern analysis of this blot suggested the tagged plasmid had inserted into a *Bam*HI site in the genome.

20 **Example 45: Characterization of morphological mutant *Aspergillus oryzae* DEBY 5-7.1 rescued locus pHowB571**

 The *Aspergillus oryzae* DEBY5-7.1 rescued locus pHowB571 containing 600 bp was sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ ID NO:71) shown in Figure 37 showed no homology to any published sequences.

25 To test if a deletion occurred during tagging, a Southern blot was prepared and analyzed according to the method described in Example 14 using genomic DNA from *Aspergillus oryzae* HowB430 digested with *Sph*I, *Sal*I and *Bam*HI. Probes from either end of the rescued tagged plasmid pSMO571 were generated by PCR. Probe 7 was generated with primer 970936 and primer 970937 shown below. Probe 8 was generated with primer 970938 and primer 970939 shown below. The primers were synthesized with an Applied
30 Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions. The template for the PCR labeling reaction was 10 ng of pSMO571. PCR cycles and conditions were as described in Example 20.

970936: 5'-CTTCCTCATAAACCACCC-3' (SEQ ID NO:72)

970937: 5'-AACTGACAGGACAAGACC-3' (SEQ ID NO:73)

970938: 5'-GACTTGCATCACTTCCTC-3' (SEQ ID NO:74)

970939: 5'-TGAAGCTGAGAGTAGGTG-3' (SEQ ID NO:75)

5 The results showed identical banding patterns from both probes suggesting no deletions had occurred. PCR was used to verify that no deletions had occurred using the method described in Example 20. A total of 500 ng of genomic DNA from *Aspergillus oryzae* HowB430 was used as template with primer 970936 and primer 970939. A 550 bp product was amplified as predicted. Southern data from genomic DNA obtained from the
10 tagged mutant *Aspergillus oryzae* DEBY5-7.1 digested with *Bam*HI, hybridized to the *Nhe*I fragment of *pyrG* suggested that the tagged plasmid had inserted into a *Bam*HI site in the genome. Southern blot conditions are described as above.

Southern and PCR analysis demonstrated the tagged plasmid had inserted directly into a *Bam*HI site. Cloning with TOPO pCRII vector and subsequent sequencing according to
15 Example 2 of the PCR product generated using *Aspergillus oryzae* HowB430 genomic DNA with primers from the rescued ends of *Aspergillus oryzae* DEBY5-7.1 confirmed this result.

Example 46: Characterization of morphological mutant *Aspergillus oryzae* DEBY8-10.1 pSMO810

20 The *Aspergillus oryzae* DEBY8-10.1 rescued locus pSMO810 containing 750 bp was sequenced according to the method described in Example 2. The nucleic acid sequence (SEQ ID NO:76) shown in Figure 38 showed no homology to any published sequences.

Probes from either end of the rescued plasmid pSMO810 were generated by PCR. Probe 9 was generated with primer 970854 and primer 970855 shown below. Probe 10 was
25 generated with primer 970856 and primer 970857 shown below. The primers were synthesized with an Applied Biosystems Model 394 DNA/RNA Synthesizer according to the manufacturer's instructions. The template for the PCR labeling reaction was 10 ng of pSMO810. The PCR reaction and conditions were as described in Example 20.

970854: 5'-GTTTCGGTATTGTCCTG-3' (SEQ ID NO:77)

30 970855: 5'-ACAGGTGAACAAGTGG-3' (SEQ ID NO:78)

970856: 5'-CGACCAAAGTACACAAGC-3' (SEQ ID NO:79)

970857: 5'-CTTTCCTCTTGGACACAC-3' (SEQ ID NO:80)

Southern analysis was performed as described in Example 14. Southern analysis of genomic DNA from *Aspergillus oryzae* HowB430 digested with *Sph*I, *Sal*I, and *Bam*HI hybridized independently with probes 9 and 10 suggested no deletions had occurred during the insertion of the tagged plasmid. PCR using 500 ng of *Aspergillus oryzae* HowB430 genomic DNA as template with primers 090854 and 090856 amplified a 500 bp product as expected with no deletions. Southern analysis of genomic DNA prepared from mutant *Aspergillus oryzae* DEBY8-10.1, digested with *Eco*RI, and probed to the *Nhe*I fragment of *pyrG* suggested that the plasmid integrated into an *Eco*RI site in the genome. Cloning with TOPO pCRII vector and subsequent sequencing according to Example 2 of the PCR product generated using *Aspergillus oryzae* HowB430 genomic DNA with primers from the rescued ends of *Aspergillus oryzae* DEBY8-10.1 confirmed this result.

Example 47: Screening on a poor carbon source for high producers

Eighteen pools of *Aspergillus oryzae* HowB430 transformants, 11 generated with *Hind*III digested pDSY81 and in the presence of *Hind*III, and 7 generated with *Sal*I digested pDSY81 and in the presence of *Sal*I (see Example 5) were screened on poor carbon sources to identify mutants which were high producers of lipase. Glycerol was used as poor carbon source, since the expression of lipase is very low on glycerol, but a number of other carbon sources, *e.g.*, xylose, sucrose, and polyols such as mannitol and sorbitol could be used in a similar way.

The primary 96-well plate screen was performed as described in Example 7, but with GLY25 medium composed of 100 ml of 10% yeast extract, 100 ml of 25% glycerol, 100 ml of 2% urea per liter diluted 50-fold. Lipase assays were performed as described in Example 7.

Mutants of interest were then inoculated directly into 24-well plates containing the same medium as above and grown 6 days at 34°C and 100 rpm. Cultures were then assayed for lipase activity as described in Example 7, and mutants of interest were plated on COVE plates to produce spores, spread on PDA plates to produce single colonies, and then 4 single isolates of each mutant were grown on CM-glycerol agar (as CM-1 agar, but maltose was replaced by glycerol as carbon source) to produce spores for inoculation of 24-well plates as above.

After the 24-well plates, 10 transformants were identified for further evaluation in shake flasks. The shake flasks contained 50 ml of medium at pH 6.5 composed of 1 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 1 g/l K_2SO_4 , 15 g of KH_2PO_4 , 0.25 ml of trace metals solution, 0.7 g of yeast extract, 3 ml of 50% urea, 2 ml of 15% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, and 2% carbon source (either maltose, glucose, sucrose, or glycerol). The shake flasks containing 50 ml of medium in a 125 PP flask were inoculated with 0.5 ml G1-gly overnight culture, incubated at 34°C and 200 rpm, and sampled after 2 and 3 days. Lipase activity was measured as above.

The results are shown in Table 12 where lipase production by *Aspergillus oryzae* HowB430 grown on glycerol as carbon source is normalized to 1.0.

10

Table 12

<u>Strain</u>	<u>Pool</u>	<u>Glycerol</u>	<u>Maltose</u>	<u>Glucose</u>	<u>Sucrose</u>
HowB430	NA	1.0	152	44	1.1
HINL880.1	Hin-5	1.5	182	49	10
HINL895.3	Hin-20	8.4	140	38	11
15 HINL918.4	Hin-24	5.7	163	45	8.8
SALL587.4	Sal-4	1.6	178	89	22
SALL591.2	Sal-4	1.3	232	126	33
SALL631.2	Sal-5	16.6	72	35	25
SALL631.3	Sal-5	18.3	67	42	29
20 SALL664.2	Sal-15	6.1	70	79	9.2
SALL683.3	Sal-16	1.6	186	77	20
SALL692.2	Sal-16	2.7	148	63	24

The relative expression of lipase responded differently to different carbon sources, suggesting that the regulation of the lipase expression was altered in these transformants.

25

Example 48: Screening for α -cyclopiazonic acid mutants

The pools e1 - e26 from the *EcoRI* library described in Example 5 was used in screening for α -cyclopiazonic acid negative strains.

The spore number in the vials containing the different pools was determined by counting an appropriate dilution in a haemocytometer and a dilution series was constructed in such a way that approximately 30 - 50 spore derived colonies were present on each 9 cm screening plate. The screening medium was composed per liter of 30 g of mannitol, 10 g

of glucose, 10 g of succinic acid, 3 g of Casamino acids, 1 g of KH_2PO_4 , 0.3 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 0.2 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 100 μl of Triton X100, and 20 g of Difco Bacto Agar. The pH was adjusted to 5.6 with 14% NH_4OH before autoclaving. The ferrous ion forms a red complex with α -cyclopiazonic acid. This complex is seen on the reverse side of the colonies.

5 Approximately 2000 - 2500 colonies were screened from each pool. Colonies with no red coloration on the reverse side after 7 days incubation at 34°C were reisolated on the screening medium and incubated for 7 days at 34°C . Ten colonies originating from 6 different pools exhibited non-red reverse coloration and were subsequently inoculated onto Cove-N slants made as follows: 20 ml of Cove salt solution, 4.2 g of NaNO_3 , and 60 g of
10 glucose were dissolved in deionised water and the volume made up to 1000 ml. The medium was solidified by 2% Difco Bacto Agar.

Five ml of a spore suspension, made from a Cove slant by adding 10 ml of aqueous 0.01% Tween 80, was inoculated into 500 ml baffled shake flasks containing 100 ml of MDU1B shake flask medium. At the time of inoculation, 1.3 ml of 50% sterile filtered urea
15 was added to each shake flask. The shake flasks were incubated at 250 rpm for 5 days at 34°C .

Ten μl of supernatant from the 5 day old shake flask cultures were applied to the opposite edges of a 20 cm x 20 cm TLC plate (Merck Silica Gel 60). The plate was then run for 15 minutes in a chloroform:acetone:propan-2-ol (85:15:20) solvent system (CAP)
20 allowed to dry, turned around and the opposite side was run in a toluene: ethyl acetate: formic acid (5:4:1) solvent system (TEF) for 15 minutes. The plate was allowed to dry thoroughly for 1 hour in a fume hood before spraying with Ehrlich reagent (2 g of 4-dimethylaminobenzaldehyde in 85 ml of 96% ethanol plus 15 ml of 37% hydrochloric acid). α -Cyclopiazonic acid was seen as bluish-violet mushroom shaped spots with a typical low
25 R_f value in the CAP solvent system (a neutral system) whereas the acidic TEF solvent system yielded a typical high R_f value prolonged smear. Solutions of 30, 15, and 7.5 ppm of α -cyclopiazonic acid (Sigma Chemical Co., St. Louis, MO) in a 1:1:1 solution of ethanol, methanol, and chloroform were used as standards.

The TLC analysis of ten putative α -cyclopiazonic acid-free transformants showed no
30 sign of α -cyclopiazonic acid. The remaining contents of the shake flasks were filtered through Miracloth and 10 ml of 0.1 M hydrochloric acid were added to 60 ml of each filtrate. The acidified filtrates were then vigorously shaken for 3- 5 minutes with 50 ml

chloroform. The bottom phases (approximately 25 ml) were each transferred to a 300 ml beaker after phase separation (3 hrs) and the chloroform allowed to evaporate. The residues were each redissolved in 300 μ l of chloroform and 10 μ l of each concentrate was analyzed by TLC as described above.

- 5 None of the 10 strain extracts contained any detectable α -cyclopiazonic acid.

DEPOSIT OF BIOLOGICAL MATERIALS

The following strains have been deposited according to the Budapest Treaty in the Agricultural Research Service Patent Culture Collection (NRRL), Northern Regional
10 Research Laboratory, 1815 University Street, Peoria, Illinois 61604, USA.

Strain	Accession Number	Deposit Date
<i>E. coli</i> HB101 pDSY109	NRRL B-21623	September 5, 1996
<i>E. coli</i> DH5 α pMT1936	NRRL B-21832	September 8, 1997
<i>E. coli</i> HB101 pDSY112	NRRL B-21622	September 5, 1996
15 <i>E. coli</i> HB101 pDSY138	NRRL B-21833	September 8, 1997
<i>E. coli</i> DH5 α pDSY162	NRRL B-21831	September 8, 1997
<i>E. coli</i> DH5 α pDSY163	NRRL B-21830	September 8, 1997
<i>E. coli</i> DH5 α pSMO1204	NRRL B-21820	September 8, 1997
<i>E. coli</i> DH5 α pSMOH603	NRRL B-21821	September 8, 1997
20 <i>E. coli</i> HB101 p4-8.1	NRRL B-21823	September 8, 1997
<i>E. coli</i> HB101 p7-14.1	NRRL B-21824	September 8, 1997
<i>E. coli</i> DH5 α pHB220	NRRL B-21825	September 8, 1997
<i>E. coli</i> DH5 α pSMO717	NRRL B-21826	September 8, 1997
<i>E. coli</i> DH5 α pSMO321	NRRL B-21827	September 8, 1997
25 <i>E. coli</i> DH5 α pHowB571	NRRL B-21828	September 8, 1997
<i>E. coli</i> DH5 α pSMO810	NRRL B-21829	September 8, 1997

The strains have been deposited under conditions that assure that access to the culture will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 C.F.R. §1.14 and
30 35 U.S.C. §122. The deposits represent a substantially pure culture of each deposited strain. The deposits are available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny are filed. However, it should be

understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

The invention described and claimed herein is not to be limited in scope by the specific embodiments herein disclosed, since these embodiments are intended as illustrations
5 of several aspects of the invention. Any equivalent embodiments are intended to be within the scope of this invention. Indeed, various modifications of the invention in addition to those shown and described herein will become apparent to those skilled in the art from the foregoing description. Such modifications are also intended to fall within the scope of the appended claims.

10 All publications mentioned herein are incorporated herein by reference for the purpose of describing and disclosing the particular information for which the publication was cited. The publications discussed above are provided solely for their disclosure prior to the filing date of the present application. Nothing herein is to be construed as an admission that the inventors are not entitled to antedate such disclosure by virtue of prior invention.

15 It is to be understood that this invention is not limited to the particular methods and compositions described as such may, of course, vary. It is also to be understood that the terminology used herein is for the purpose of describing particular embodiments only, and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims.

20 Unless defined otherwise all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which this invention belongs. Although any materials or methods similar or equivalent to those described herein can be used in the practice or testing of the present invention, the preferred methods and materials are described.

SEQUENCE LISTING

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(v) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: FastSeq For Windows Version 2.0 (EPO)

(vi) CURRENT APPLICATION DATA:

- (A) APPLICATION NUMBER: To Be Assigned
- (B) FILING DATE: 12-SEPT-1997
- (C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATGCATCTGG AAACGCAACC CTGA

24

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 24 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ATGCATTCTA CGCCAGGACC GAGC

24

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

TGGTGTACAG GGCATAAAA T

21

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 34 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATTTAAATCC AGTTGTGTAT ATAGAGGATT GTGG

34

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 30 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

ATTTAAATGA TGAGGAGCTC CCTTGTGCTG

30

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 29 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

TTAATTA ACT AGAGTCGACC CAGCCGCGC

29

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GCGGGATCCC TAGAGTAGGG GGTGGTGG

28

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 28 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

GCGGGATCCC CCCTAAGGAT AGGCCCTA

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(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3000 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

ACACGGCCTG	GACAATGAGA	CAACTCTTCT	AAAGAGTTCA	GATTTAGTAT	ATATACTAGC	60
CAGGACGGAC	TTTCCAAATA	TTATGTAAAT	TAAAGTGTCG	TTGTGAAGTG	CTACCTATAA	120
TGCTTAGTAT	GTGTATGTCT	GGATGCAACG	GGCTAATATT	ACACATCAAG	AAGTCCACTC	180
AATAAGCCAC	TGGTGCAAAT	TAAGTGAATA	CACATGTATC	TATATCCGGA	GCAATAAAAA	240
CTAACGATAA	TAATGGCAAG	GGTCACTTAT	AACAAACTCA	TACTGGACAA	AAAGTATGGA	300
GAAACAATAG	ATTAAAAAGG	TCCCCTTCTA	TATTTAATCC	TCCCGCGACG	GAGTTTCCTT	360
ATCCCGATAG	ATACGATAAG	GATCCAGGTA	ACGTCATTCC	ATCCCGTGAG	ATAAAGAGGA	420
CTCCACCTCA	ACAACTAATC	ATCAAATCTC	CAATCAATAT	CAATGAACCC	ATAACAACCTT	480
AAAAAGCTCT	CGGAAAAGTA	AAAAGAGCTT	CTATCAGCAT	ATACACCATG	GTCTCGGTG	540
GATCAAGCGG	GTCAAAGGTC	ACTCCGTACC	TGATCTACCT	TGTGTTTATC	ACAACTTTGG	600
GGCCACTTCA	ATTTCGGATAT	CATTTGGTAT	TACACGGAGC	TTGGTCTATG	CTGGAGGCTT	660
CAATACATCG	GCTGACAATA	TATTATGATA	GGCTGAGCTC	AATGCCCCCC	AGGCCGTGAT	720
AACTTGCGAG	CGGAAAAGCA	TCCATTGCGA	AACAACACGG	GGTCTCCCGC	AATGCATACC	780
TATGAACCCA	TCCCAATTCT	GCCTGGTCTC	CTCTATATAC	ACCCCTTGGGG	GCTTGCTAGG	840
GGCTCTCCTG	GCAGGTCCAG	TTTCCACCAA	GCATGGCCGC	TTGTTCCACAC	TGCGAGCGAC	900
CACCATCTTC	TTCATCCTAG	GCCCTATAGC	AGAAACATTT	GCGCCCAGTA	TACCCGTATT	960
GAGTATGGGT	AGGCTTTTAT	CTGGTGTGG	TGCGGGCGCT	TCTATCGTCG	TGGGTCCGAT	1020
ATATATCTCT	GAGATTGCTC	CTCCTAGTGC	TAAGGGTCTT	TTCGGCGCTT	TTACGCAAAAT	1080
CATGACTAAT	GTCGGTATTC	TGTTGACACA	GTCCCTTGGT	TACTTCTTGA	GTAAAGGAAG	1140
TATGTGGAGA	GTTATACTTG	CAATTGCTGG	CGCGATCGGA	TGCTTTGAGC	TTCTGGGCTT	1200
CTTCTTAGTC	CCAGAAAGCC	CCATCTGGCT	TGCAGATCAC	CAGAAAGGGA	ATGTGGCTAG	1260
ACAGGTGCTA	CAACGTATAC	GGGGCAGGGA	TGCAGACATC	GAGCCAGAGG	TTGAAGGCTG	1320
GAGAACATCT	GCAGCGCCTG	AACACAGCTC	TGGGGAAGAG	CAGTCCCTAC	TATCACCCCC	1380
ATCTGGAAT	ATGCCACCCA	AGCAACCTCC	GGTTACCATG	ATGCGAGCTA	TTACTGATTC	1440
TTTTTACCAG	CCTGCCATCA	TTGCAGTGGT	CGGAGTCATG	GTTTCCCAGC	AGTTCATCTG	1500
TGTC AACGC	ATCATCATGT	ACAGCGTTTC	CCTCTTACAG	ACCATCCTTC	CCACCACTGC	1560
AGCCCTGTTG	TCGGTGATCA	TCTCGGCTAT	CAATCTTGTA	ATCACTCTGG	CCTGCTCACC	1620
ACTACCTGAT	AAGATTGGTA	GACGCTCCTG	CCTGCTTCTA	AGTATCAGCG	GCATGGGTCT	1680
TAATTCCGTC	CTACTGGCGC	TAGCCATCTA	CTTCAACCTG	AAAGCCTTAT	CCGCCATAGC	1740
AGTTCTACTT	TTCTGTGCTT	CTTTCGCCCG	CGGCTAGGCG	CCAGTCCCTT	TCATTTTAGC	1800
CTCTGAACCT	GTTGGCCCGG	AGGCTGTCGG	CGCCGCACAG	AGCTGGGCGC	TGGGAGCGAA	1860
CTGGATTGCC	ACGTTTCATG	TGGCACAATT	TTTTCCGATG	TTAAACGATT	TGTTGGGCGG	1920
ACGAGGCAAG	ATCTACTGGA	TCTTTGCAGC	GATGGCCTGT	CTCCTCGGAA	GTTTCATCTA	1980
CTGGTGGGTG	CCGGAGACCA	AGGGGAAGGC	TAACGCCGAC	GAAGTTTGGG	GAAGGACCAA	2040
CCAGCGCAGA	CAGGACTAAT	TTTTCTGGCC	TCTTTGATTT	TTTTTTTCTG	GGCCTTACTC	2100
TGCTGCCAAC	ATTCAGATTA	TCAATTAGTA	GTCAATCTGT	GACTATCCTC	TCCGAGGGAT	2160
AGCTTGCAAA	GGTGTGACCT	CCACAGAGGA	ATCTATCGTG	TGACAGTATC	AAAGACAATA	2220
GAATAGCAAT	AATTGGTGCT	CTCTACCTAG	GAGCATTCGG	TGAGAGTGAA	AGAGTCATAC	2280
TTGCCTCGGC	TTGTTTCATCC	CAGTCGATCA	GTCAGGTTTA	GCTCGGCAGT	AAAAGCAATA	2340

CCGGTCTACT	TCCATCTTCA	AACTGTACCG	CGGAAACAAA	GAGTAAAGGA	GGGGTCATGA	2400
TACCTCTAAA	TAATGTATAA	GTCGTTGACA	ATGCTCTTTA	TCACCACCCG	TTGAAGACGT	2460
CCTTTGATGT	CTTGATCATC	ACAAGCAGGT	TGATCATCTG	CGATCGACGT	CACTTCGCAC	2520
CGCACACTGC	ATGACAAAGT	CGGGGCAGAG	GGGCCAACAG	GCCCCAAAAT	TTAGTGATTT	2580
TGAAGCAATG	TTGTTACACC	CTTTTACCCC	TCAATCCATG	ATAAGGGAAA	AAGAGATGCT	2640
GAGAGAGGGT	CACTGCCACG	CTAGACTGGA	TTGGTCCGTA	TATGCAGGTT	TATGCACGCA	2700
CAGGGGGGCT	TCGTTCTTCT	TGGCTATGCA	CTATGGATTA	GTAGGGTGTA	TTCAACCACG	2760
TAGATAGATT	GGCGTGTCCT	GTGCAGGATA	TGTAGAAGAC	AATGAGGTTT	GGGCTTTCCG	2820
AAATGAGGAA	AGAATGTTGG	ACAGATGAAA	AACGGTACTG	CTGTTGCAAG	GGGGCGCTGT	2880
TTGAGATATT	TTAAGTGCCT	GTCATGTAAT	TTTGCAACGG	TGAGACATTT	ATCTAGGGTA	2940
AAATCCAAGA	AGAACCTAGG	GAAGAGTAAA	GCCACAACGA	AGATTACGTG	AGAGGAAGAG	3000

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 488 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:10:

Met	Val	Leu	Gly	Gly	Ser	Ser	Gly	Ser	Lys	Val	Thr	Pro	Tyr	Leu	Ile
1				5					10					15	
Tyr	Leu	Val	Phe	Ile	Thr	Thr	Leu	Gly	Pro	Leu	Gln	Phe	Gly	Tyr	His
			20					25					30		
Leu	Ala	Glu	Leu	Asn	Ala	Pro	Gln	Ala	Val	Ile	Thr	Cys	Glu	Arg	Lys
			35				40					45			
Ser	Ile	His	Ser	Thr	Thr	Thr	Arg	Gly	Leu	Pro	Gln	Cys	Ile	Pro	Met
			50			55					60				
Asn	Pro	Ser	Gln	Phe	Gly	Leu	Val	Ser	Ser	Ile	Tyr	Thr	Leu	Gly	Gly
65					70					75				80	
Leu	Leu	Gly	Ala	Leu	Leu	Ala	Gly	Pro	Val	Ser	Thr	Lys	His	Gly	Arg
				85					90					95	
Leu	Phe	Thr	Leu	Arg	Ala	Thr	Thr	Ile	Phe	Phe	Ile	Leu	Gly	Pro	Ile
			100					105					110		
Ala	Glu	Thr	Phe	Ala	Pro	Ser	Ile	Pro	Val	Leu	Ser	Met	Gly	Arg	Leu
			115				120					125			
Leu	Ser	Gly	Val	Gly	Ala	Gly	Ala	Ser	Ile	Val	Val	Gly	Pro	Ile	Tyr
			130				135					140			
Ile	Ser	Glu	Ile	Ala	Pro	Pro	Ser	Ala	Lys	Gly	Leu	Phe	Gly	Ala	Phe
145					150					155				160	
Thr	Gln	Ile	Met	Thr	Asn	Val	Gly	Ile	Leu	Leu	Thr	Gln	Ser	Leu	Gly
				165					170					175	
Tyr	Phe	Leu	Ser	Lys	Gly	Ser	Met	Trp	Arg	Val	Ile	Leu	Ala	Ile	Ala
			180					185					190		
Gly	Ala	Ile	Gly	Cys	Leu	Glu	Leu	Leu	Gly	Leu	Phe	Leu	Val	Pro	Glu
			195				200					205			
Ser	Pro	Ile	Trp	Leu	Ala	Asp	His	Gln	Lys	Gly	Asn	Val	Ala	Arg	Gln
			210			215					220				
Val	Leu	Gln	Arg	Ile	Arg	Gly	Arg	Asp	Ala	Asp	Ile	Glu	Pro	Glu	Val
225					230					235				240	
Glu	Gly	Trp	Arg	Thr	Ser	Ala	Ala	Pro	Glu	His	Ser	Ser	Gly	Glu	Glu
				245					250					255	
Gln	Ser	Leu	Leu	Ser	Pro	Pro	Ser	Gly	Asn	Met	Pro	Pro	Lys	Gln	Pro
			260					265					270		
Pro	Val	Thr	Met	Met	Arg	Ala	Ile	Thr	Asp	Ser	Phe	Tyr	Arg	Pro	Ala
			275				280						285		
Ile	Ile	Ala	Val	Val	Gly	Val	Met	Val	Ser	Gln	Gln	Phe	Thr	Gly	Val
			290			295					300				
Asn	Ser	Ile	Ile	Met	Tyr	Ser	Val	Ser	Leu	Leu	Gln	Thr	Ile	Leu	Pro
305					310					315				320	
Thr	Thr	Ala	Ala	Leu	Leu	Ser	Val	Ile	Ile	Ser	Ala	Ile	Asn	Leu	Val
				325					330					335	

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Ile Thr Leu Ala Cys Ser Pro Leu Pro Asp Lys Ile Gly Arg Arg Ser
      340      345      350
Cys Leu Leu Leu Ser Ile Ser Gly Met Gly Leu Asn Ser Val Leu Leu
      355      360      365
Ala Leu Ala Ile Tyr Phe Asn Leu Lys Ala Leu Ser Ala Ile Ala Val
      370      375      380
Leu Leu Phe Val Ala Ser Phe Ala Ala Gly Leu Gly Pro Val Pro Phe
385      390      395      400
Ile Leu Ala Ser Glu Leu Val Gly Pro Glu Ala Val Gly Ala Ala Gln
      405      410      415
Ser Trp Ala Leu Gly Ala Asn Trp Ile Ala Thr Phe Ile Val Ala Gln
      420      425      430
Phe Phe Pro Met Leu Asn Asp Leu Leu Gly Gly Arg Gly Lys Ile Tyr
      435      440      445
Trp Ile Phe Ala Ala Met Ala Cys Leu Leu Gly Ser Phe Ile Tyr Trp
      450      455      460
Trp Val Pro Glu Thr Lys Gly Lys Ala Asn Ala Asp Glu Val Trp Gly
465      470      475      480
Arg Thr Asn Gln Arg Arg Gln Asp
      485

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(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 488 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:11:

```

Met Ala Glu Thr Glu Arg Leu Met Pro Asn Gly Gly Ser Arg Glu Thr
 1      5      10      15
Lys Pro Leu Ile Thr Gly His Leu Ile Leu Gly Thr Ile Val Ala Cys
      20      25      30
Leu Gly Ser Ile Gln Tyr Gly Tyr His Ile Ala Glu Leu Asn Ala Pro
      35      40      45
Gln Glu Phe Leu Ser Cys Ser Arg Phe Glu Ala Pro Asp Glu Asn Ile
      50      55      60
Ser Tyr Asp Asp Thr Trp Val Gly Gln His Gly Leu Lys Gln Cys Ile
65      70      75      80
Ala Leu Thr Asp Ser Gln Tyr Gly Ala Ile Thr Ser Ile Phe Ser Ile
      85      90      95
Gly Gly Leu Phe Gly Ser Tyr Tyr Ala Gly Asn Trp Ala Asn Arg Tyr
      100      105      110
Gly Arg Lys Tyr Val Ser Met Gly Ala Ser Ala Met Cys Met Val Ser
      115      120      125
Ser Leu Leu Leu Phe Phe Ser Asn Ser Tyr Leu Gln Leu Leu Phe Gly
      130      135      140
Arg Phe Leu Val Gly Met Ser Cys Gly Thr Ala Ile Val Ile Thr Pro
145      150      155      160
Leu Phe Ile Asn Glu Ile Ala Pro Val Glu Trp Arg Gly Ala Met Gly
      165      170      175
Ser Met Asn Gln Val Ser Ile Asn Leu Gly Ile Leu Leu Thr Gln Thr
      180      185      190
Leu Ala Leu Lys Tyr Ala Asp Ser Tyr Asn Trp Arg Trp Leu Leu Phe
      195      200      205
Ser Gly Ser Val Ile Ala Val Ala Asn Ile Leu Ala Trp Leu Lys Val
      210      215      220
Asp Glu Ser Pro Arg Trp Leu Val Ser His Gly Phe Val Ser Glu Ala
225      230      235      240
Glu Thr Ala Leu Phe Lys Leu Arg Pro Gly Thr Tyr Gln Gln Ala Lys
      245      250      255

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Gln Glu Ile Gln Asp Trp Gln Arg Ser His Gly His Asn Arg Asp Pro
      260      265      270
Glu Ser Ser Glu Glu Thr His Ser Gly Pro Thr Leu Trp Gln Tyr Val
      275      280      285
Thr Asp Pro Ser Tyr Lys Lys Pro Arg Thr Val Ile Leu Ala Ile Leu
      290      295      300
Ser Cys Gln Gln Phe Cys Gly Ile Asn Ser Ile Ile Phe Tyr Gly Val
305      310      315      320
Lys Val Ile Gly Lys Ile Leu Pro Asp Tyr Ser Ile Gln Val Asn Phe
      325      330      335
Ala Ile Ser Ile Leu Asn Val Val Val Thr Leu Ala Ala Ser Ala Ile
      340      345      350
Ile Asp His Val Gly Arg Arg Pro Leu Leu Leu Ala Ser Thr Thr Val
      355      360      365
Met Thr Ala Met Ser Leu Leu Ile Ser Val Gly Leu Thr Leu Ser Val
      370      375      380
Ser Phe Leu Leu Val Thr Ala Thr Phe Val Tyr Ile Ala Ala Phe Ala
385      390      395      400
Ile Gly Leu Gly Pro Ile Pro Phe Leu Ile Ile Gly Glu Leu Ser Tyr
      405      410      415
Pro Gln Asp Ala Ala Thr Ala Gln Ser Phe Gly Thr Val Cys Asn Trp
      420      425      430
Leu Ala Thr Phe Ile Val Gly Tyr Leu Phe Pro Ile Gly His Gly Leu
      435      440      445
Met Gly Gly Tyr Val Phe Ala Ile Phe Ala Ala Ile Ala Ala Met Phe
      450      455      460
Ala Thr Tyr Val Tyr Lys Arg Val Pro Glu Thr Lys Gly Lys Thr Thr
465      470      475      480
Tyr Ser Glu Val Trp Ala Gly Tyr
      485

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(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 524 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:12:

```

Met Thr Glu Asp Lys Val Thr Gly Thr Leu Val Phe Thr Val Ile Thr
 1      5      10      15
Ala Val Leu Gly Ser Phe Gln Phe Gly Tyr Asp Ile Gly Val Ile Asn
      20      25      30
Ala Pro Gln Gln Val Ile Ile Ser His Tyr Arg His Val Leu Gly Val
      35      40      45
Pro Leu Asp Asp Arg Lys Ala Ile Asn Asn Tyr Val Ile Asn Ser Thr
      50      55      60
Asp Glu Leu Pro Thr Ile Ser Tyr Ser Met Asn Pro Lys Pro Thr Pro
65      70      75      80
Trp Ala Glu Glu Glu Thr Val Ala Ala Ala Gln Leu Ile Thr Met Leu
      85      90      95
Trp Ser Leu Ser Val Ser Ser Phe Ala Val Gly Gly Met Thr Ala Ser
      100      105      110
Phe Phe Gly Gly Trp Leu Gly Asp Thr Leu Gly Arg Ile Lys Ala Met
      115      120      125
Leu Val Ala Asn Ile Leu Ser Leu Val Gly Ala Leu Leu Met Gly Phe
      130      135      140
Ser Lys Leu Gly Pro Ser His Ile Leu Ile Ile Ala Gly Arg Ser Ile
145      150      155      160
Ser Gly Leu Tyr Cys Gly Leu Ile Ser Gly Leu Val Pro Met Tyr Ile
      165      170      175

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Gly Glu Ile Ala Pro Thr Ala Leu Arg Gly Ala Leu Gly Thr Phe His
      180      185      190
Gln Leu Ala Ile Val Thr Gly Ile Leu Ile Ser Gln Ile Ile Gly Leu
      195      200      205
Glu Phe Ile Leu Gly Asn Tyr Asp Leu Trp His Ile Leu Leu Gly Leu
      210      215      220
Ser Gly Val Arg Ala Ile Leu Gln Ser Leu Leu Leu Phe Phe Cys Pro
      225      230      235      240
Glu Ser Pro Arg Tyr Leu Tyr Ile Lys Leu Asp Glu Glu Val Lys Ala
      245      250      255
Lys Gln Ser Leu Lys Arg Leu Arg Gly Tyr Asp Asp Val Thr Lys Asp
      260      265      270
Ile Asn Glu Met Arg Lys Glu Arg Glu Glu Ala Ser Ser Glu Gln Lys
      275      280      285
Val Ser Ile Ile Gln Leu Phe Thr Asn Ser Ser Tyr Arg Gln Pro Ile
      290      295      300
Leu Val Ala Leu Met Leu His Val Ala Gln Gln Phe Ser Gly Ile Asn
      305      310      315      320
Gly Ile Phe Tyr Tyr Ser Thr Ser Ile Phe Gln Thr Ala Gly Ile Ser
      325      330      335
Lys Pro Val Tyr Ala Thr Ile Gly Val Gly Ala Val Asn Met Val Phe
      340      345      350
Thr Ala Val Ser Val Phe Leu Val Glu Lys Ala Gly Arg Ser Leu
      355      360      365
Phe Leu Ile Gly Met Ser Gly Met Phe Val Cys Ala Ile Phe Met Ser
      370      375      380
Val Gly Leu Val Leu Leu Asn Lys Phe Ser Trp Met Ser Tyr Val Ser
      385      390      395      400
Met Ile Ala Ile Phe Leu Phe Val Ser Phe Phe Glu Ile Gly Pro Gly
      405      410      415
Pro Ile Pro Trp Phe Met Val Ala Glu Phe Phe Ser Gln Gly Pro Arg
      420      425      430
Pro Ala Ala Leu Ala Ile Ala Ala Phe Ser Asn Trp Thr Cys Asn Phe
      435      440      445
Ile Val Ala Leu Cys Phe Gln Tyr Ile Ala Asp Phe Cys Gly Pro Tyr
      450      455      460
Val Phe Phe Leu Phe Ala Gly Val Leu Leu Ala Phe Thr Leu Phe Thr
      465      470      475      480
Phe Phe Lys Val Pro Glu Thr Lys Gly Lys Ser Phe Glu Glu Ile Ala
      485      490      495
Ala Glu Phe Gln Lys Lys Ser Gly Ser Ala His Arg Pro Lys Ala Ala
      500      505      510
Val Glu Met Lys Phe Leu Gly Ala Thr Glu Thr Val
      515      520

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(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 584 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:13:

```

Met Gly Ile His Ile Pro Tyr Leu Thr Ser Lys Thr Ser Gln Ser Asn
  1           5           10           15
Val Gly Asp Ala Val Gly Asn Ala Asp Ser Val Glu Phe Asn Ser Glu
      20      25      30
His Asp Ser Pro Ser Lys Arg Gly Lys Ile His Ile Glu Ser His Glu
      35      40      45
Ile Gln Arg Ala Pro Ala Ser Asp Asp Glu Asp Arg Ile Gln Ile Lys
      50      55      60

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Pro	Val	Asn	Asp	Glu	Asp	Asp	Thr	Ser	Val	Met	Ile	Thr	Phe	Asn	Gln
65					70					75					80
Ser	Leu	Ser	Pro	Phe	Ile	Ile	Thr	Leu	Thr	Phe	Val	Ala	Ser	Ile	Ser
				85					90					95	
Gly	Phe	Met	Phe	Gly	Tyr	Asp	Thr	Gly	Tyr	Ile	Ser	Ser	Ala	Leu	Ile
			100					105					110		
Ser	Ile	Gly	Thr	Asp	Leu	Asp	His	Lys	Val	Leu	Thr	Tyr	Gly	Glu	Lys
		115					120					125			
Glu	Ile	Val	Thr	Ala	Ala	Thr	Ser	Leu	Gly	Ala	Leu	Ile	Thr	Ser	Ile
		130				135					140				
Phe	Ala	Gly	Thr	Ala	Ala	Asp	Ile	Phe	Gly	Arg	Lys	Arg	Cys	Leu	Met
145					150					155					160
Gly	Ser	Asn	Leu	Met	Phe	Val	Ile	Gly	Ala	Ile	Leu	Gln	Val	Ser	Ala
				165					170					175	
His	Thr	Phe	Trp	Gln	Met	Ala	Val	Gly	Arg	Leu	Ile	Met	Gly	Phe	Gly
			180					185					190		
Val	Gly	Ile	Gly	Ser	Leu	Ile	Ala	Pro	Leu	Phe	Ile	Ser	Glu	Ile	Ala
		195					200					205			
Pro	Lys	Met	Ile	Arg	Gly	Arg	Leu	Thr	Val	Ile	Asn	Ser	Leu	Trp	Leu
	210				215						220				
Thr	Gly	Gly	Gln	Leu	Val	Ala	Tyr	Gly	Cys	Gly	Ala	Gly	Leu	Asn	Tyr
225				230						235					240
Val	Asn	Asn	Gly	Trp	Arg	Ile	Leu	Val	Gly	Leu	Ser	Leu	Ile	Pro	Thr
			245						250					255	
Ala	Val	Gln	Phe	Thr	Cys	Leu	Cys	Phe	Leu	Pro	Asp	Thr	Pro	Arg	Tyr
			260					265					270		
Tyr	Val	Met	Lys	Gly	Asp	Leu	Ala	Arg	Ala	Thr	Glu	Val	Leu	Lys	Arg
		275					280					285			
Ser	Tyr	Thr	Asp	Thr	Ser	Glu	Glu	Ile	Ile	Glu	Arg	Lys	Val	Glu	Glu
	290					295					300				
Leu	Val	Thr	Leu	Asn	Gln	Ser	Ile	Pro	Gly	Lys	Asn	Val	Pro	Glu	Lys
305				310						315					320
Val	Trp	Asn	Thr	Ile	Lys	Glu	Leu	His	Thr	Val	Pro	Ser	Asn	Leu	Arg
			325						330					335	
Ala	Leu	Ile	Ile	Gly	Cys	Gly	Leu	Gln	Ala	Ile	Gln	Gln	Phe	Thr	Gly
			340					345					350		
Trp	Asn	Ser	Leu	Met	Tyr	Phe	Ser	Gly	Thr	Ile	Phe	Glu	Thr	Val	Gly
		355					360					365			
Phe	Lys	Asn	Ser	Ser	Ala	Val	Ser	Ile	Ile	Val	Ser	Gly	Thr	Asn	Phe
	370					375					380				
Ile	Phe	Thr	Leu	Val	Ala	Phe	Phe	Ser	Ile	Asp	Lys	Ile	Gly	Arg	Arg
385				390						395					400
Thr	Ile	Leu	Leu	Ile	Gly	Leu	Pro	Gly	Met	Thr	Met	Ala	Leu	Val	Val
			405						410					415	
Cys	Ser	Ile	Ala	Phe	His	Phe	Leu	Gly	Ile	Lys	Phe	Asp	Gly	Ala	Val
			420					425					430		
Ala	Val	Val	Val	Ser	Ser	Gly	Phe	Ser	Ser	Trp	Gly	Ile	Val	Ile	Ile
		435					440					445			
Val	Phe	Ile	Ile	Val	Phe	Ala	Ala	Phe	Tyr	Ala	Leu	Gly	Ile	Gly	Thr
	450					455					460				
Val	Pro	Trp	Gln	Gln	Ser	Glu	Leu	Phe	Pro	Gln	Asn	Val	Arg	Gly	Ile
465					470					475					480
Gly	Thr	Ser	Tyr	Ala	Thr	Ala	Thr	Asn	Trp	Ala	Gly	Ser	Leu	Val	Ile
			485						490					495	
Ala	Ser	Thr	Phe	Leu	Thr	Met	Leu	Gln	Asn	Ile	Thr	Pro	Ala	Gly	Thr
			500					505					510		
Phe	Ala	Phe	Phe	Ala	Gly	Leu	Ser	Cys	Leu	Ser	Thr	Ile	Phe	Cys	Tyr
		515					520					525			
Phe	Cys	Tyr	Pro	Glu	Leu	Ser	Gly	Leu	Glu	Leu	Glu	Glu	Val	Gln	Thr
	530					535					540				
Ile	Leu	Lys	Asp	Gly	Phe	Asn	Ile	Lys	Ala	Ser	Lys	Ala	Leu	Ala	Lys
545					550					555					560
Lys	Arg	Lys	Gln	Gln	Val	Ala	Arg	Val	His	Glu	Leu	Lys	Tyr	Glu	Pro
			565						570					575	
Thr	Gln	Glu	Ile	Ile	Glu	Asp	Ile								
			580												

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CTGCCGTCGA AGGTGTCCAA G

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(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:15:

ATTGTGGCCC CTATGTGGAT T

21

(2) INFORMATION FOR SEQ ID NO:16:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4700 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:16:

ACAGCGACTG	GGATGGTGAA	TATCTGAGCG	ATAGCCCCCG	ACACAGCACC	AAGGGTAAGC	60
TCCATAGCGG	TTCCAGGTGG	CTTAGATACG	CTCTTCGATG	CCATATAAAG	GCTTCTAACC	120
ACGCTGTACC	AGTAGAAGTA	CGCAAAGTTG	GTGAGGCCA	CGCCAAGCAA	AGAACCAACC	180
ATCCCGGAAT	ATAAACCTTC	AATTCCCTCT	TTCTCCACAA	TCTTGTGAT	GGCATCTAGG	240
GTCGACTCGT	AATGTACTAC	ATCTCCGCTT	TTCGATTGAG	GTGCGTTCTT	CACTTGGACT	300
TGAAGTTTGG	TTTTGACACT	AAAGATTAGG	AACGAGTCAG	TCTCAGTCTA	CACCACTAAG	360
CCAAGTGGCA	AGGCTATGTA	CGCACAGGTC	CAGTGGATAG	ACGATAGCAT	TTGCGAGAAC	420
AGCACCAGTT	GCACCTGCGA	CAGCACTACC	CCAAGGGGAG	AGCGCGGGTT	TCGATTGGCC	480
GGCCATTATG	CAGGATGAGC	TAAAGTGCC	CTGCCAATTC	CGTCAGAAAG	AATAGTATAA	540
GAGCACAAAT	ACTGGTAAAA	CCAAGACCGG	CGAATGAGGC	AGGACTCTGT	GCGATTCCGG	600
GGGTTTAGCG	TTGCGCTTGA	AGGCTTACCG	GATCGACTGA	TAGAAAAGTT	GAATGCCGAG	660
TAAGGTGAAA	AGACCCGAGC	TCCCAAGCAG	CAACAGTAAG	TGGAAGAGCT	TAAGGATAGA	720
AAAAATAAAT	TAGGATTAAG	AAAAAAGAA	GAACCTCAAG	ACTGGTCACA	CAGTCCCGGC	780
ATCCTGAACG	TAAATGCGG	GAAGGATAGA	GTGCGCAGGG	CCAGGGCAGT	TGCACCTCGG	840
CGCTCTGGTT	TGCGCATGAC	GAAATGAGCC	GAGGTTCTGT	TTTTGGAGGC	CAATTTCTGA	900
ACACCGACCT	TCGAATTCCC	GTTCTCTCCC	ACCACACGCG	TAGTGAATGA	TCCAGCAAGC	960
ATACTTGGTG	TTGTTTTGAC	CTCATTCCAC	TGCGTGTGAA	TTAGCATTAA	TTTAAGTTTA	1020
TGATTAACAG	TCAATTGCTA	TACGCGAAAA	TCATCATCGT	CTTGATTGGC	CCTTCATAAA	1080
ACTTGACAAG	GAAGTTTGAT	CGACCTCGGA	TGTCGCGCTT	TCGGAAATTT	CACGGAGCCC	1140
TTCCGACGGG	TCACAAGCAA	GGTGTCTGAC	TGTCTCGTTT	AGTCGGATAG	ACGCTAGTTG	1200
AAGTGTATG	CCTATCGCGG	GGAAGATCTC	GGAGTGTAC	GGTGTGTTGA	GATCCCAGGC	1260
GCTCGTCAAA	ATACTGCCCC	GCCTGCCAGT	ATGTCTAGAC	CGAACGCCTC	AGCCGAGAAG	1320
TCCTTTATAA	CTCAGGCACT	GGTACTTGAC	CCTTTTTTTT	TATGGTTTTT	TGTTTTCTTC	1380
TTGTTACACC	TTATTTTTCT	TCTTCTCGTT	TTTTGTAGAT	AATACTGACC	ACTGGCTAGA	1440
AAGCCGAGCG	GGATGTATCG	TCCGCCACTT	CTCAAAGGCA	AGCTTTAGAA	GCTGCCATTG	1500
ATGCTGCTGA	ACACTATATG	AAAGCCTTAA	ATCTGGCATC	TGTTTCAGAA	GACAAACATG	1560
CATTGGATGC	AAAGTGTAAA	GAATGGCTCA	CAAGAGCGGA	AAAGATCAAA	GAATCTAAGG	1620
ACTGGCAAGC	TGCTGCCCGT	TTCCATGACA	AAACTGTTCC	AGAGCCACGG	TTGCCTGTAT	1680
CTACTCGTAA	GCTCACCACA	CGGGAGGAGA	TCATTCTGCT	AGAGGGAGCC	AAGTTGAATG	1740

GCTTCATATT	CCCTCCATGG	TCCACCTCCC	CAGGCTCTGA	CGAGTTCAAA	CGAGAGGATG	1800
GTGAATCCCC	GTTTACGTAA	GTTCTGGTGG	TCTGCATCGT	CAATGTTGCA	TGTATACCCA	1860
GATGACTGCT	GGATATTCTA	ACCGATAACA	GCGACAAACC	CGATCTTCAT	CTATCTTATC	1920
CTCAAAGGAA	AGTTTTTGAT	GGCTGGAAC	GACCTTCCGA	GCTTCTCGCG	AAAGACACGG	1980
AAGATGTGTA	CACAAAGGTG	GTTCTGTGTA	TGTCTGTTCC	AGGAAAGACA	GATCTAGTCC	2040
AGGATATGCT	GACGGACTGT	TCTGTGCTTG	CTAGCCTTTG	TGCTACTACG	TCAATGCTAG	2100
AACGCGGCCA	GTGTACTGTA	AGAAGATTGA	TCCCTTCCGG	CTGACCTGCA	TGGTTCGCTG	2160
TGACTAATAG	GTGTAGCATT	TTCTTCCAAT	GATATACCTT	AGCCGGGGGA	GCTCTCAGCC	2220
TTACCGGTCA	GGCAAGTATA	TATTTGCTTT	TTATTTCAAT	GGGTGCTTCC	GGAAAAGTCAT	2280
CATTGACGAC	CGTTTGCCAT	CGTCTAAGAC	ATCAAGATCA	CTCCACGTGA	TCGACCGGAA	2340
AAATCCCAAT	TTCTTTTGGC	CGGCGCTCGT	AGAGAAGGCG	TATTTGAAAT	TGCGCGGAGG	2400
CTATGATTTT	CCCAGGAAGCA	ATTCCGGGAC	AGATCTCTGG	GTGCTGACAG	GTTGGATTCC	2460
CGAGCAAGTC	TTTCTCCATA	ATGACGATGT	GAATGCGGAC	CAGCTCTGGA	AGCGACTTTA	2520
CAGATCCTTT	CACCAAGGAG	ATGTTCTCTT	GACTATAGGT	ACCGGTGAAC	TCAGTGAAG	2580
GGAACAAAGA	GAAGTAGGCC	TCGTGAGTGA	GCAATGATTAT	GCTATTCTGG	ATATGAAGGA	2640
ATCTAAAGGT	CGCCGACAAT	TACTCGTGAA	AAACCTTGG	GCTGGAGCAG	ATACTGCCCC	2700
CGGCGACAAT	GGAAGCCTCT	CTGCATCGCA	GGATTTACCC	CATAACCCGC	CCTCATTGTA	2760
GCCGGGTACC	TTTTGGATGG	ATTGCGAAAA	GCTGCTTCAA	CATTTTGAAA	ACCTCTATTT	2820
GAATTGGAAC	CCTGAGATTT	TCAAATACCG	CGAAGACGTC	CACCTTACGT	GGGACCTCAA	2880
CAACGGGAGA	GGTGTAGCCG	GCTGTTTTGT	GAATAACCCG	CAGTTCGCAG	TGTCAACCGA	2940
GAACGGTGGG	ATTGCTGGT	TACTTCTAGG	CAAGCATTTT	AGAACAACAG	GGCAGCCGGA	3000
ACGACCTCTT	GACGAATACC	AAGCGAATGA	GGAGTCGGCT	TTTATAAGCA	TATATGTCTT	3060
TAACGCAGAT	GGCAAACGGG	TCTCTTTGAG	TGATGGGGCT	CTACATCGTG	GCCCCATATG	3120
GGATTCCCCT	AATACGCTCA	TGAGGTTAGA	GATGCCCCCC	AGAACAACAT	ACACAGTCGT	3180
GGTCTCCGAG	CAATCACTGC	CATCTTTGAA	TCAAACTTTT	ACTTTGTCTG	CGTTCTCTAC	3240
CTGCCCTGTA	CGGATGGCAA	AAGCCCAAGA	TAAATACATG	TGTGTCAGGA	AGATTCAAGG	3300
GTCTTTGACA	CCTTCGACGG	CAGGTGGGAA	TGCCGAATCT	TCTCGATATC	CACTCAACCC	3360
CCAATTTAGG	TTGGAGATAG	AGAATGACAC	AGATGTTTCA	CTCCTGCTGG	AATGCCCCAA	3420
CACGGAACCT	GCGACCCATG	TTAAGTTATT	CTGGTCCAAT	GGAAATCGTG	TGTCGCGAGT	3480
ACGCAGTCGC	GACATAATCG	CTGATAGTGG	TGACTATCGC	CGTGGTGGCT	CCCTTGTGGA	3540
AAAGAAGGCT	CTGGAACCGG	GCTCATATAC	AATCGTCTGT	TCCACATTCT	CGCCGGATCA	3600
ACTTGGCCGA	TTACGCTCT	GGGTATCCTC	CTTAGTTTCT	TGCAAGACGA	GCCCCGCTCC	3660
ACCAGAGGCA	GCAGGTCGAC	GAACGGTCAT	TTCAGATATT	GGCGTACTGC	CTCCCGGGAG	3720
AGACCGAATG	TTAGCTTCTC	TGCAAGTGCC	GCGGCTTACG	AGGATCAAGC	TCATCACCCG	3780
AAGTAGGCAA	TCCATCATCG	GGAGCCATCC	TGTTGGACCC	TCGCCCCGTT	TAATGACAGT	3840
GGAGCTCGGG	CAAGGGCCAT	ACAAACAGAT	CCTGGCGACT	TCGGAAGATG	GAACTCACAG	3900
TGATGCTGTA	TCGGGGGTAC	GTGTTGAGGA	CTTTGACTTG	CAGCCTGGGC	TAGAGGAGAG	3960
TGGTGGTATT	TGGATTGTTA	TTGAGAGGAT	TGGGGGTCCT	GGAGGGCAGG	TAGAGGACCA	4020
CTTTGAGGTG	GAAGCTTTGG	CTGAAGAGAG	GGTTGAGATT	GGGGAGTGGA	TACTTGAAGA	4080
TGCTTGATCT	CTTATCCTGC	AGAAGCTCTG	AAGCTCTGGA	CGGTCTTAGT	TGAGCTTTTTT	4140
TGATCGTCTG	TGTGATTAGC	ACGTTAGAGT	AGAAGAGCGG	AAACAATGAT	AGACATGAAT	4200
TTCTCTTATT	GTCTCTATTG	GCCAGAAGAG	AAAGAAGGCA	TTAAATCAAT	ACATTAAAAG	4260
CAAGAGTTTA	TCTATAGATA	CCGAGCAGCC	TCAGGATTTG	AGCTGAGGTT	TGTCGCGATC	4320
GCGACCGCCA	AAATGGAGTT	AGCTTGCTTT	ACTCCGCATA	AATTAAATCC	TCGGCTCGGG	4380
GCCCCGAATC	CCCTCCCAGG	TGCTTTCAAC	GTCATCCCGT	TTGCTGTGTG	CATTGTGCCT	4440
CCCCACCTTT	AACAATTGGA	GCTCTGTCCA	AGGACAAATC	CTTATTCTCG	CGGCCTCCAT	4500
GGCGACTAAT	ATTCTGTCTG	CTCTGAAGTC	TGCGGACATT	GGGCGCTTTG	CCGTCAGAGC	4560
AGCTCAGCTT	GAACGTGTAA	AGCCCCGTGG	CGCCTACTGG	TGTGAGTATT	GTGTGATTAT	4620
ACCCAGTACG	AACTACGAGC	TGATGAGCGG	CCTCTGCTGA	TCGAGGCAAA	CTTCTGGATC	4680
GTCAACCAGA	TTATTGAGAA					4700

(2) INFORMATION FOR SEQ ID NO:17:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 854 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:17:

Met Ser Arg Pro Asn Ala Ser Ala Gln Lys Ser Phe Ile Thr Gln Ala
 1 5 10 15

Leu	Lys	Ala	Glu	Arg	Asp	Val	Ser	Ser	Ala	Thr	Ser	Gln	Arg	Gln	Ala			
			20					25					30					
Leu	Glu	Ala	Ala	Ile	Asp	Ala	Ala	Glu	His	Tyr	Met	Lys	Ala	Leu	Asn			
		35				40						45						
Leu	Ala	Ser	Val	Gln	Lys	Asp	Lys	His	Ala	Leu	Asp	Ala	Lys	Cys	Lys			
	50					55					60							
Glu	Trp	Leu	Thr	Arg	Ala	Glu	Lys	Ile	Lys	Glu	Ser	Lys	Asp	Trp	Gln			
65					70					75					80			
Ala	Ala	Ala	Arg	Phe	His	Asp	Lys	Thr	Val	Pro	Glu	Pro	Arg	Leu	Pro			
				85					90					95				
Val	Ser	Thr	Arg	Lys	Leu	Thr	Thr	Arg	Glu	Glu	Ile	Ile	Leu	Leu	Glu			
			100					105					110					
Gly	Ala	Lys	Leu	Asn	Gly	Phe	Ile	Phe	Pro	Pro	Trp	Ser	Thr	Ser	Pro			
		115				120						125						
Gly	Ser	Asp	Glu	Phe	Lys	Arg	Glu	Asp	Gly	Glu	Ser	Pro	Phe	Thr	Asp			
	130					135					140							
Lys	Pro	Asp	Leu	His	Leu	Ser	Tyr	Pro	Gln	Arg	Lys	Val	Phe	Asp	Gly			
145					150					155					160			
Trp	Lys	Arg	Pro	Ser	Glu	Leu	Leu	Ala	Lys	Asp	Thr	Glu	Asp	Val	Tyr			
				165					170					175				
Thr	Lys	Val	Val	Pro	Val	Met	Ser	Val	Pro	Gly	Lys	Thr	Asp	Leu	Val			
			180					185					190					
Gln	Asp	Met	Leu	Thr	Asp	Cys	Ser	Val	Val	Ala	Ser	Leu	Cys	Ala	Thr			
		195					200					205						
Thr	Ser	Met	Leu	Glu	Arg	Gly	Gln	Cys	Thr	His	Phe	Leu	Pro	Met	Ile			
	210					215					220							
Tyr	Pro	Ser	Arg	Gly	Ser	Ser	Gln	Pro	Ser	Pro	Ser	Gly	Lys	Tyr	Ile			
225					230					235					240			
Phe	Arg	Phe	Tyr	Phe	Asn	Gly	Cys	Phe	Arg	Lys	Val	Ile	Ile	Asp	Asp			
				245				250						255				
Arg	Leu	Pro	Ser	Ser	Lys	Thr	Ser	Arg	Ser	Leu	His	Val	Ile	Asp	Arg			
			260					265					270					
Lys	Asn	Pro	Asn	Phe	Leu	Trp	Pro	Ala	Leu	Val	Glu	Lys	Ala	Tyr	Leu			
		275					280					285						
Lys	Leu	Arg	Gly	Gly	Tyr	Asp	Phe	Pro	Gly	Ser	Asn	Ser	Gly	Thr	Asp			
	290					295					300							
Leu	Trp	Val	Leu	Thr	Gly	Trp	Ile	Pro	Glu	Gln	Val	Phe	Leu	His	Asn			
305					310					315					320			
Asp	Asp	Val	Thr	Gly	Asp	Gln	Leu	Trp	Lys	Arg	Leu	Tyr	Arg	Ser	Phe			
				325					330					335				
His	Gln	Gly	Asp	Val	Leu	Leu	Thr	Ile	Gly	Thr	Gly	Glu	Leu	Thr	Glu			
			340					345					350					
Arg	Glu	Gln	Arg	Glu	Leu	Gly	Leu	Val	Ser	Glu	His	Asp	Tyr	Ala	Ile			
		355					360					365						
Leu	Asp	Met	Lys	Glu	Ser	Lys	Gly	Arg	Arg	Gln	Leu	Val	Lys	Asn				
	370					375					380							
Pro	Trp	Ala	Gly	Ala	Asp	Thr	Ala	Pro	Gly	Asp	Asn	Gly	Ser	Leu	Ser			
385					390					395					400			
Ala	Ser	Gln	Asp	Leu	Pro	His	Asn	Pro	Pro	Ser	Phe	Glu	Pro	Gly	Thr			
				405					410					415				
Phe	Trp	Met	Asp	Cys	Glu	Lys	Leu	Leu	Gln	His	Phe	Glu	Asn	Leu	Tyr			
			420					425					430					
Leu	Asn	Trp	Asn	Pro	Glu	Ile	Phe	Lys	Tyr	Arg	Glu	Asp	Val	His	Phe			
	435						440					445						
Thr	Trp	Asp	Leu	Asn	Asn	Gly	Arg	Gly	Val	Ala	Gly	Cys	Phe	Val	Asn			
	450					455					460							
Asn	Pro	Gln	Phe	Ala	Val	Ser	Thr	Glu	Asn	Gly	Gly	Ile	Val	Trp	Leu			
465					470					475					480			
Leu	Leu	Gly	Lys	His	Phe	Arg	Thr	Thr	Gly	Gln	Pro	Glu	Arg	Pro	Leu			
				485					490					495				
Asp	Glu	Tyr	Gln	Ala	Asn	Glu	Glu	Ser	Ala	Phe	Ile	Ser	Ile	Tyr	Val			
			500					505					510					
Phe	Asn	Ala	Asp	Gly	Lys	Arg	Val	Ser	Leu	Ser	Asp	Gly	Ala	Leu	His			
		515					520					525						
Arg	Gly	Pro	Tyr	Val	Asp	Ser	Pro	Asn	Thr	Leu	Met	Arg	Leu	Glu	Met			
	530					535					540							

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Pro Pro Arg Thr Thr Tyr Thr Val Val Val Ser Glu Gln Ser Leu Pro
545                               550                               555
Ser Leu Asn Gln Asn Phe Thr Leu Ser Ala Phe Ser Thr Cys Pro Val
                               565                               570
Arg Met Ala Lys Ala Gln Asp Lys Tyr Met Cys Val Arg Lys Ile Gln
                               580                               585
Gly Ser Trp Thr Pro Ser Thr Ala Gly Gly Asn Ala Glu Ser Ser Arg
                               595                               600
Tyr Pro Leu Asn Pro Gln Phe Arg Leu Glu Ile Glu Asn Asp Thr Asp
610                               615                               620
Val Ser Leu Leu Leu Glu Cys Pro Asn Thr Glu Leu Ala Thr His Val
625                               630                               635
Lys Leu Phe Trp Ser Asn Gly Asn Arg Val Ser Arg Val Arg Ser Arg
                               645                               650
Asp Ile Ile Ala Asp Ser Gly Asp Tyr Arg Arg Gly Gly Ser Leu Val
660                               665                               670
Glu Lys Lys Ala Leu Glu Pro Gly Ser Tyr Thr Ile Val Cys Ser Thr
675                               680                               685
Phe Ala Pro Asp Gln Leu Gly Arg Phe Thr Leu Trp Val Ser Ser Leu
690                               695                               700
Val Pro Cys Lys Thr Ser Pro Leu Pro Pro Glu Ala Ala Gly Arg Arg
705                               710                               715
Thr Val Ile Ser Asp Ile Gly Val Leu Pro Pro Gly Arg Asp Arg Met
725                               730                               735
Leu Ala Ser Leu Gln Val Pro Arg Leu Thr Arg Ile Lys Leu Ile Thr
740                               745                               750
Arg Ser Arg Gln Ser Ile Ile Gly Ser His Pro Val Gly Pro Ser Pro
755                               760                               765
Val Leu Met Thr Val Glu Leu Gly Gln Gly Pro Tyr Lys Gln Ile Leu
770                               775                               780
Ala Thr Ser Glu Asp Gly Thr His Ser Asp Ala Val Ser Gly Val Arg
785                               790                               795
Val Glu Asp Phe Asp Leu Gln Pro Gly Leu Glu Glu Ser Gly Gly Ile
805                               810                               815
Trp Ile Val Ile Glu Arg Ile Gly Gly Pro Gly Gly Gln Val Glu Asp
820                               825                               830
His Phe Glu Val Glu Ala Leu Ala Glu Glu Arg Val Glu Ile Gly Glu
835                               840                               845
Trp Ile Leu Glu Asp Ala
850

```

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 842 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:18:

```

Met Ser Arg Thr Ser Ser Ala Pro Ser Gln Lys Ser Leu Ile Ser Arg
1                               5                               10                               15
Ala Leu Lys Ala Glu Arg Asp Val Ile Thr Ala Ser Ser Gln Ser Gln
20                               25                               30
Ala Leu Asp Ala Ala Ile Asp Ala Ala Glu His Tyr Met Lys Ala Leu
35                               40                               45
Ala Leu Thr Ser Ser Ser Lys Asp Arg Asn Val Leu Asp Ala Lys Cys
50                               55                               60
Lys Glu Trp Leu Thr Arg Ala Glu Lys Ile Lys Gly Ser Glu Asp Trp
65                               70                               75                               80
Arg Ser Val Ala Gln Ser Arg Arg Ser Arg Leu Arg Thr Pro Ala Ser
85                               90                               95

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Thr Arg Lys Leu Thr Thr Arg Glu Asp Ile Ile Leu Leu Gln Gly Ala
 100 105 110
 Lys Leu Asn Gly Phe Ile Phe Pro Pro Trp Lys Ala Glu Pro Ser Leu
 115 120 125
 Thr Glu Phe Glu Thr Gly Thr Asn Gly Asp Val Leu Phe Thr Asp Lys
 130 135 140
 Pro Asp Leu His Leu Ser Asn Leu Gln Arg Asp Ile Phe Ala Gly Trp
 145 150 155 160
 Lys Arg Pro His Glu Leu Leu Ser Gly Gln Val Asp Asp Ala Gly Met
 165 170 175
 Pro Leu Asn Pro Val Met Thr Val Ser Gly Asn Thr Asp Leu Val Gln
 180 185 190
 Asp Val Leu Thr Asp Cys Ser Val Ala Ser Leu Cys Ala Thr Thr
 195 200 205
 Ser Arg Ser Glu Arg Gly Leu Asp Asp Thr Leu Leu Pro Ile Val Tyr
 210 215 220
 Pro Cys Ile His Asn Ser Met Lys Ser Asp Ile Ser Pro Ser Gly Lys
 225 230 235 240
 Tyr Ile Phe Arg Phe Tyr Phe Asn Gly Cys Phe Arg Lys Val Val Ile
 245 250 255
 Asp Asp Arg Leu Pro Ser Ser Lys Thr Ser Arg Ser Leu Tyr Met Ile
 260 265 270
 Asp Arg Asn His Arg Asn Phe Met Trp Pro Ala Leu Val Glu Lys Ala
 275 280 285
 Tyr Leu Lys Leu Arg Gly Gly Tyr Glu Phe Pro Gly Ser Asn Ser Gly
 290 295 300
 Thr Asp Leu Trp Val Leu Thr Gly Trp Ile Pro Glu Gln Val Phe Leu
 305 310 315 320
 His Ser Asp Glu Val Thr Ala Asp Gln Ile Trp Ser Asp Leu Phe Lys
 325 330 335
 Ser Phe His Ser Gly Asp Val Leu Leu Thr Ile Gly Thr Gly Lys Leu
 340 345 350
 Thr Glu Arg Glu Gln Lys Glu Leu Gly Leu Val Ser Glu His Asp Tyr
 355 360 365
 Ala Ile Leu Asp Met Lys Glu Leu Lys Gly Arg Arg Gln Phe Leu Ile
 370 375 380
 Lys Asn Pro Trp Ala Gly Thr Asp Ala Val Tyr Pro Ala Leu Phe Ala
 385 390 395 400
 Asp Pro Gly Pro Phe Pro Asn Ser Pro Phe Leu Ser Pro Gly Thr Phe
 405 410 415
 Trp Met Asp Cys Glu Met Val Leu Gln Asn Phe Glu Asn Leu Tyr Leu
 420 425 430
 Asn Trp Asn Pro Gly Ile Phe Ala Tyr Gln Glu Asp Ile His Phe Thr
 435 440 445
 Trp Asp Leu Ser Thr Gly Lys Gly Met Ala Gly Cys Phe Val Lys Asn
 450 455 460
 Pro Gln Phe Ser Val Tyr Thr Glu Arg Gly Gly Val Val Trp Leu Leu
 465 470 475 480
 Leu Gly Arg His Leu Arg Thr Ile Glu Ser Arg Ala Ser Glu Glu Asp
 485 490 495
 Glu Arg Phe Gly Phe Ile Ser Ile Tyr Val Phe Lys Gly Gly Lys Arg
 500 505 510
 Val Ala Leu Ser Asp Gly Ala Leu His Arg Gly Pro Tyr Val Asp Ser
 515 520 525
 Pro Asn Thr Leu Met Lys Leu Asp Val Pro Pro Arg Ser Thr Tyr Thr
 530 535 540
 Ala Val Val Ser Glu Glu Ser Leu Pro Arg Val Ser Gln Asn Phe Thr
 545 550 555 560
 Ile Ser Ala Phe Ser Asp Ser Pro Val Arg Ile Ser His Ala Pro Asn
 565 570 575
 Lys Tyr Ile Cys Val Thr Lys Val Gln Gly Ser Trp Thr Pro Thr Thr
 580 585 590
 Ala Gly Gly Asn Ala Glu Ser Ala Arg Tyr Ser Leu Asn Pro Gln Phe
 595 600 605
 Ser Ile Val Leu Ser Asp Pro Thr Asp Ile Ser Ile Val Leu Glu Pro
 610 615 620

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Ser Asp Gln Glu Leu Ala Thr His Val Lys Leu Phe Trp Ser Gly Gly
625          630          635          640
Lys Arg Ile Ala Arg Val Arg Ser Arg Asp Ile Val Ala Asp Ser Gly
          645          650          655
Asp Tyr Arg Arg Gly Gly Ser Leu Val Glu Lys Gln Asp Leu Asp Pro
          660          665          670
Gly Glu Tyr Thr Ile Val Val Ser Thr Phe Ala Pro Asp Gln Tyr Gly
          675          680          685
Ser Phe Thr Leu Trp Val Ser Thr Asn Ile Thr Cys Glu Val Thr Gln
          690          695          700
Leu Pro Ser Glu Ala Ala Gly Arg Arg Ala Val Leu Ser Asp Ile Gly
705          710          715          720
Val Leu Leu Pro Gly Gln Asp Arg Met Leu Ala Pro Leu Thr Thr Pro
          725          730          735
Arg Leu Thr Arg Val Lys Leu Ile Ala Arg Ser Arg Glu Ser Arg Ile
          740          745          750
Gly Asn Arg Pro Val Gly Pro Ser Pro Leu Leu Met Thr Val Glu Leu
          755          760          765
Gly Gln Gly Pro Tyr Lys Glu Ile Leu Ala Thr Ser Glu Asp Gly Asp
          770          775          780
His Ser Asp Ser Ile Ser Gly Val Arg Val Glu Asp Phe Asp Leu Gln
785          790          795          800
Pro Gly Leu Glu Glu Arg Gly Gly Val Trp Ile Val Leu Glu Arg Ile
          805          810          815
Gly Gly Leu Ala Val Lys Trp Lys Ile Ile Ser Lys Trp Lys Leu Trp
          820          825          830
Glu Lys Arg Glu Trp Arg Leu Gly Asn Gly
          835          840

```

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 38 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:19:

```

Gly Ile Leu Leu Ala Gly Ser Ala Asn Ser Lys Tyr Ala Phe Leu Gly
1          5          10          15
Ser Leu Arg Ser Thr Ala Gln Leu Ile Ser Tyr Glu Leu Ile Leu Ser
          20          25          30
Ser Val Ile Leu Leu Val
          35

```

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 371 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:20:

```

Met Phe Tyr Ser Leu Thr Ile Ile Ser Ile Leu Glu Val Leu Leu Val
1          5          10          15
Leu Val Pro Ser Leu Leu Ala Val Ala Tyr Val Thr Val Ala Glu Arg
          20          25          30

```

Lys Thr Met Ala Ser Met Gln Arg Arg Leu Gly Pro Asn Ala Val Gly
 35 40 45
 Tyr Leu Gly Leu Leu Gln Ala Phe Ala Asp Ala Leu Lys Leu Leu Leu
 50 55 60
 Lys Glu Tyr Val Ala Leu Thr Gln Ala Asn Met Thr Leu Phe Phe Leu
 65 70 75 80
 Gly Pro Val Ile Thr Leu Ile Phe Ser Leu Leu Gly Tyr Ala Val Ile
 85 90 95
 Pro Tyr Gly Pro Ser Leu Val Ile Gln Asp Val Asn Leu Gly Ile Leu
 100 105 110
 Tyr Met Leu Ala Val Ser Ser Leu Ala Thr Tyr Gly Ile Leu Leu Ala
 115 120 125
 Gly Trp Ser Ala Asn Ser Lys Tyr Ala Phe Leu Gly Ser Leu Arg Ser
 130 135 140
 Ala Ala Gln Leu Ile Ser Tyr Glu Leu Val Leu Ser Ser Ala Ile Leu
 145 150 155 160
 Leu Val Ile Met Leu Thr Gly Ser Phe Asn Leu Gly Val Asn Thr Glu
 165 170 175
 Ser Gln Arg Ala Val Leu Phe Val Leu Pro Leu Leu Pro Ile Phe Ile
 180 185 190
 Ile Phe Phe Ile Gly Ser Ile Ala Glu Thr Asn Arg Ala Pro Phe Asp
 195 200 205
 Leu Ala Glu Ala Glu Ser Glu Leu Val Ser Gly Phe Met Thr Glu His
 210 215 220
 Ala Ala Val Val Phe Val Phe Phe Phe Leu Ala Glu Tyr Gly Ser Ile
 225 230 235 240
 Val Leu Met Cys Ile Leu Thr Ser Ile Leu Phe Leu Gly Gly Tyr Leu
 245 250 255
 Phe Ile Asn Leu Lys Asp Val Phe Asn Ile Leu Asp Phe Val Tyr Ser
 260 265 270
 Asn Leu Phe Ile Phe Glu Ile Asn Trp Met Val Ser Glu Arg Ser Tyr
 275 280 285
 Thr Glu Asp Phe Phe Asn Asn Tyr Lys Ser Ile Leu Glu Gly Trp Leu
 290 295 300
 Tyr Gly Trp Ile Ile Gly Leu Lys Ser Ser Ile Met Ile Phe Ile Phe
 305 310 315 320
 Ile Leu Gly Arg Ala Ser Phe Pro Arg Ile Arg Tyr Asp Gln Leu Met
 325 330 335
 Gly Phe Cys Trp Thr Val Leu Leu Pro Ile Ile Phe Ala Leu Ile Ile
 340 345 350
 Leu Val Pro Cys Ile Leu Glu Ser Phe Tyr Ile Leu Pro Trp Asn Leu
 355 360 365
 Asn Leu Phe
 370

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 36 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:21:

GGTTGCATGC TCTAGACTTC GTCACCTTAT TAGCCC

36

(2) INFORMATION FOR SEQ ID NO:22:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 40 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:22:

TTCGCGCGCA TCAGTCTCGA GATCGTGTGT CGCGAGTACG 40

(2) INFORMATION FOR SEQ ID NO:23:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:23:

GATCTCGAGA CTAGTGC GCG CGAACAGACA TCACAGGAAC C 41

(2) INFORMATION FOR SEQ ID NO:24:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:24:

CAACATATGC GGCCGCGAAT TCACTTCATT CCCACTGCGT GG 42

(2) INFORMATION FOR SEQ ID NO:25:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 6800 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:25:

TATTCTGCTA GTAGTTAGAT CTACTGAGGG GGTATAACTC TTCGGTAGCC GGTGCGTATG 60
 CAGTTGTATT GTGACTGATA GATGAGAAGA AGGGCGTTTA CTGGTTGCAA GAGAATGATG 120
 TTATGCTTTG CATATAAGAG ATGGAATTAT TACTGATATG TGTCTAAAAT TACAATGATT 180
 TGTTTTACTG AAAGTGAAGT TAATGCCAGT TTGGATGTAG CCGCGTAGGT GATACTTAAT 240
 CCGGACCAAA TTATGAGACC TCGATAGTAC AATGCTATTG GACTTGTAAG ATAATAGTCT 300
 AGTTCTTCAT AGACAAACCT GAAAAAGGAA GGACTCGAAG TCATTTTCGAC AAGCCATAGT 360
 ATGATTAGGC ATCTAGGAGC TGAACGGAAA CGTCGTATGC GAGAGGGCCA GAACCAAAAT 420
 ACGACAGTAG GAGGATTCTT ACCCCTTGGT ATATATGGAA ACCGGTTTTA AAGACTGGGC 480
 GTCCTCGTAC CTCACAGTCG AGCATGCCAG ACGTTGGCGC ACACATTCCC CAGATCGGCT 540
 GGAGCCTATC GCAATGCAAC CGTACCCACC CGGCGTNTAC TATATCTGAG CATTCTTGCT 600
 GCAAGTCGTG GAGGTTGTTC CAGCCAATGA GGTCCGTTGA GCTTCTTTTT CCGATATGGT 660
 GATAGCTGAC AGTGTACTAG TGTCGACTGC ACAGGTGCCT CGCGGAAATG TGAGATCGGA 720
 GCATGTCTTG CTTAGAGGAC CGAAGTATGG ACGAATGTCC GCCATCTTGA AGAATGGTGA 780
 CTACCGGAGG ATTGTCTCCA GCTCAGGACT ATGTATATGC CACGATGCCA CGGCTTTGTG 840
 TGATAGAGTT CAAGAGCCGA GATTTACAAC GGTGATTACT ATCGCAGATA TCAGCACAGT 900
 ACCGGCAAGA TGGTCAACTG GAGCAACATA CTACCCACAT TAACCGCCTT CATCGCTTTT 960
 ATCCTGGGCA TGCTATGCCT CTTTGCAGGA ACGAAAACAA ACCTTTTATT AGATACAGAT 1020
 GTCTTCACGG TGTGTGAGCC TCCGTACAC TGCTTGTAC GAGCGGGGAC TAACGACAGG 1080
 CGAAGATATA TACGACAAGT ATAAGCAATG GCACGGGGAT GCAGGACTTC TACTCGATAT 1140
 ATGTCATGTC TTAGTGTGAG GGATTCCTGC ATGCAGAAAA TCGAAACCTA ACCGGATGCT 1200
 CACACCCGTC ACTACTGTTC TCCTTCAATG CGACAGAAGC GTTAACGAAA GATGCTGGCA 1260
 ACAACACCTC GTTATCCAGC CTGGGATGGC CGAGTTCCAT CACCGATGAT CTACGCACGT 1320
 TCGGTGCTAC CAGCCAGAGT ATGGGTGTCT TCTACTGTAT TGGGATAGGA TTAGCGGGAC 1380
 TGGCGGTTTT GGAACGATTG TGGTTCGTGA TCGCGAAAGG GCCGAGACAG ACGGTGTGTAG 1440

AAGTTTCTTC	TCTTATGGTA	GGCTCCATTG	AACGTTCTAG	ATCCCGTCCG	GTACTAAGAA	1500
GGCCACACAG	TCAGTTTTCAC	TATGCTCAGC	ATACCGTCTA	TCATCGCAAC	GGTCGTTGCC	1560
TTACAATTTG	TGAGCCTCAT	CAATCGTCAC	GGAGAGGAGT	CTGGTGTGAC	AGCGAGATAT	1620
GGACATCAAT	TTTTAGGAAT	GACGTGGGCA	GCCGTGCGGT	TGTTGTCTGGT	CGGAAGCACT	1680
GTCAGTTTAC	TGACGGTATT	GGTGGACCGC	AACCGATCGG	CAGACCAGTA	TGAACCGGTG	1740
GCAGAACCGA	AGACGGTGGC	CGAGGATTCTG	GACTCGGTAG	CGTCGAACCA	GAAGGGGGAC	1800
TAAGAAAGGA	ACAGAATGGA	GAGGAATAAT	CATAAGAGAA	AAAAAGGGGG	AAATTAACCA	1860
AAGCAGGAAA	AGTAGGGAAA	AAAAAAAGAA	GAAGACCGGA	GAAAGCCAAG	GAAAGGGAAC	1920
GAATCGGGAG	GAGTGTCTTCT	TGTTTGCAAA	TACGTTGGAT	TGGAGCCCCA	TATTGAATAT	1980
ACTCCGTACT	GTAGTCGAAA	GAAAGGACAA	GAGCCCCACC	AAACCTCGAC	CGTTCCATAG	2040
CAGAAATTCC	ATGACTATCT	GTTAATATTT	TCTGATCGAT	GATGTTGTAA	CAGTGGTTGA	2100
AGTGGACCTT	ATTTTTTCGCA	ATACAATAGC	AGCATGGCTT	GTGGGAAGGG	GTCTTGAGAT	2160
CATAGTCATA	GTGTAAGAAA	AAATTGGGTC	TCCC CGGGAT	GGCGACGTCA	CGAGTGGACC	2220
CGGGAAGAGG	TCTCGCCAAA	TGAGGCAACC	CGGTTCCCTC	CGTTCCAACC	GCTATGCCTG	2280
CGATTCTATC	AATGATAGTG	GGTCACTCGA	TGGATTCAAG	GGATGTCAAA	TGATGACGTT	2340
AAGTTGACTA	CGACTGTTGC	TGTCATGCAC	TGGAAATTTA	TCGGGAGGAA	TTGACCAGTC	2400
TTATTGCGGG	AGGAGGGAGT	GAAGAGACTA	GGAGTCGTTT	AGTTCAGTCT	TGCAGGAAAT	2460
CCACTCGGGG	ATGAGCAGGG	GTTAACCTCA	TGGACGACTG	AACTACTCCG	TACAGGACGG	2520
AGTACAGAAT	GTCGAATTCA	CCCGTTCGGC	TAAAGATGAG	TCCGTTTTAT	GGTCACCTAG	2580
CGGAGTGCAA	CCCCGACTGG	ATAACTGTTA	GGAAAAGAGA	GAATTAGGAT	CGGGTGACAA	2640
TTGGGTCTCT	AAGTTCCTGC	GTAACGTGAT	ACCGAATCAA	ATCCATGACC	CTATCGCCAG	2700
GACGATCACG	GAATGGTCCG	GATCAGAAAT	TCTGAGGTTT	TGGTTGGATT	GATGACCTGA	2760
ACTAATACCC	AATATCATGA	CGAAAACCAA	TCCCCTCATT	TCCTGTTTTT	GCACGGGAAT	2820
AGCCACAATT	TCCCCCCCCC	CCCCCCCCC	CCAGAAAACC	GAAATGAAGT	CTGAGCCCTC	2880
CGGAAGACTG	CGTTCCTAGC	CCCCATGTGT	TTAGTTGATA	CTATTCCCAT	AGGCACGTTT	2940
CCCCCTTCC	CCACTTGAGT	TCCACCAGTC	GTGAATGAGG	AGGTTCCATT	CTCGCCGAGA	3000
GTTTGGTTTC	TTTGCCGTTT	CATACAAGGC	GTCACTGCAT	CATTCTTCC	CTTTTCATTC	3060
ACCCCTCTCT	TGTCCACCAT	CGTGAAATGT	TTCTGTAGTA	CATTTAATAA	ATACCCCTCG	3120
TTACCCCTCT	TTCTGTTTTCC	CAAGAAATCA	ACATCATCAT	CAACAACAAC	AACAACAATC	3180
ACCCTCCCAC	TTCACAGGTT	CTCTTTCTGA	TATCCATCCT	TCTGTCTCTC	ATCTACTACC	3240
ACTACTTTCA	TATACTCTCT	TCTATCTTAC	TTTATCACCA	TCACAACCTT	CTTCCCCATT	3300
CTTGTTTTCA	CCCAACATCA	ATATATTACC	GTTGTCAACC	ATCCATCATG	GGTAAAAAGG	3360
CTATCCAGTT	TGGCGGTGGA	AACATTGGCC	GTGGCTTTGT	GGCTGAGTTT	CTCCACGCTG	3420
CCGGCTATGA	AGTCGTCTTC	ATTGATGTCA	TGGATAGCGT	CATCAACTCT	TTGCAACAGA	3480
CCCCGTCGTA	CGACGTCACG	GAGGTTCAGC	AAGAGGGTGA	AAGCACCAG	ACCATCACCA	3540
ACTATCGCGC	CATCAACTCC	AAGACGCATG	AGGCCGAGC	CGTTCAGGAG	ATCGCATCGG	3600
CAGATGTGGT	TACCTGTGCT	GTCGGTCCCA	ACATCCTTAA	GTTTCATCGC	CCAGTCATTG	3660
CCAAAGGTAT	TGATGCGCGC	ACCGAAGAGA	GACCCGTGGC	TGTGATCGCC	TGTGAGAACG	3720
CTATCGGCGC	TACAGATACC	TTGCACGGCT	ACATCAAGCA	GCACACCAAC	CCTGACCGTC	3780
TGGAGACCCT	CTCTGAGCGT	GCCCGTTTTG	CCAACTCGGC	TATCGACCGC	ATCGTCCCA	3840
ACCAGCCCCC	GAACAGTGGT	CTCAATGTTT	GCATCGAGAA	GTTCTACGAG	TGGGCCGTGG	3900
AGAAGACTCC	ATTTGGCGAA	TGGGGTCACC	CCGACATCCC	TGCCATCCAC	TGGGTGGACC	3960
ACCTCGAACC	TTACATCGAA	CGCAAGCTCT	TCACCGTCAA	CACTGGCCAT	GCTACCACCG	4020
CCTACTATGC	TCACAAGCGT	GGCAAGAAGA	TGATCGCCGA	GGCCCTCGAA	GACCCAGAGA	4080
TCCGCGAGAC	TGTGCACAAG	GTGCTCGAGG	AGACTGCTTC	CCTCATTGTA	TCCAAGCATG	4140
AGATCTCGGA	GCAGGAGCAG	AAGGAATACG	TTGACAAGAT	TGTCAGCCGT	ATCTCCAACC	4200
CCTATCTCGA	GGACAACGTT	GAGCGTGTGG	GACGTGCTCC	TCTCCGCAAA	CTGTCTCGCA	4260
AGGAACGGTT	CATTGGACCT	GCTTCGCAGC	TCCGAGAGCG	CGGCCAGAAG	TTGATGCTC	4320
TCCTGGGCGC	CATCGAGATG	GCTCTTCGCT	TCCAGAACGT	CCCAGGCGAC	GAGGAGAGTT	4380
CCGAGCTTGC	TCGCATTTTG	AAGGAGAACT	CGGCCGAGGA	TGCCACCTCG	CAGCTCACCG	4440
GATTGGAGAA	AGACCACCCA	CTCTACTCTC	ATGTGGTTGA	GCGTGTGTCC	ACGGTCCAGC	4500
AAGGCTCCAA	ATCAGTGCTG	TGATTCTCGA	TCGTTTTCCA	CACCACCACA	CTCCTTTTAA	4560
TCACCAGAAA	ACGAAGGGTT	CCGAGTCCAT	CACCAATATG	GATCGCCCGA	GGGATATTGG	4620
ATCTGATATC	AAACTGTTCT	GTCCGCTGGC	CGGCATGAA	CTGCATGGGA	TACGGCGAAC	4680
ATATGAAATA	ACCCCAATT	CCCATAAGTA	TTACATATTA	TGGAACCACA	GCCGGTGTCT	4740
GTAAATGTGC	GTTCAACTCG	AAGATGGCCG	ATGCAATCGG	CCCGTAGGGT	ATATGGTCTG	4800
GCGCCACCTC	GGCCGCCGGC	TTCCCCCTTT	TTATAGATGT	GGCGAATAAA	ACACCGGATG	4860
TTTTGTGTGT	CAGGGGAATG	GTGGCAGTGG	TGTTATGAGT	CATTGTGAAG	TGAGTAGTGA	4920
GTAGATTTGG	TGGGGATTTT	CATAGATGGT	GGTTTGAAGG	TCTTGGGTTT	CTGGGGTTTA	4980
TCCGCGTATA	TTCTGCTAGT	AGTTAGATCT	ACTGAGGGGG	TATAACTCTT	CGGTAGCCGG	5040
TGCGTATGCA	GTTGTATTGT	GACTGATAGT	TGAGAAGAAG	GGCGTTTACT	GGTTGCAAGA	5100
GAATGATGTT	ATGCTTTGCA	TATAAGAGAT	GGAATTATTA	CTGATATGTG	TCTAAAATTA	5160
CAATGATTTG	TTTTACTGAA	ACTGAGTATA	ATGCCAGTTT	GGATGTAGCC	GCGTAGGTGA	5220
TACTTAATCC	GGACCAAATT	ATGAGACCTC	GATAGTACAA	TGCTATTGGA	CTTGTAAGAT	5280
AATAGTCTAG	TTCTTCATAG	ACAAACCTGA	AAAAGGAAGG	ACTCGAAGTC	ATTTGACAAA	5340
GCCATAGTAT	GATTAGGCAT	CTAGGAGCTG	AACGGAAAACG	TCGTATGCGA	GAGGGCCAGA	5400
ACCAAATAC	GACAGTAGGA	GGATTCCCTAC	CCCTTGGTAT	ATATGGAAAC	CGGTTTTTAA	5460

GACTGGGCGT	CCTCGTACCT	CACAGTCGAG	CATGCCAGAC	GTTGGCGCAC	ACATTCCCCA	5520
GATCGGCTGG	AGCCTATCGC	AATGCAACCG	TACCCACCCG	GCGTCTACTA	TATCTGAGCA	5580
TTCTTGCTGC	AAGTCGTGGA	GGTTGTTCCA	GCCAATGAGG	TCCGTTGAGC	TTCTTTTTTC	5640
GATATGGTGA	TAGCTGACAG	TGTACTAGTG	TCGACTGCAC	AGGTGCCTCG	CGGAAATGTG	5700
AGATCGGAGC	ATGTCCTGCT	TCAGCGACCG	AAGTATGGAC	GAATGTCCGC	CATCTTGAAG	5760
AATGGTGACT	ACCGGAGGAT	TGTCTCCAGC	TCAGGACTAT	GTATATGCCA	CGATGCCACG	5820
GCTTTGTGTG	ATAGAGTTCA	AGAGCCGAGA	TTTACAACGG	TGATTACTAT	CGCAGATATC	5880
AGCACAGTAC	CGGCAAGATG	GTCAACTGGA	GCAACATACT	ACCCACATTA	ACCGCCTTCA	5940
TCGCTTTTAT	CCTGGGCATG	CTATGCCTCT	TTGCAGGAAC	GAAAACAAAC	CTTTTATTAG	6000
ATACAGATGT	CTTCACGGTG	TGTGAGCCTC	CGTCACACTT	GCTTGTACGA	GCGGGGACTA	6060
ACGACAGGCG	AAGATATATA	CGACAAGTAT	AAGCAATGGC	ACGGGGGATG	GGGACTTCTA	6120
CTCGATATAT	GTCATGTCTT	ACTGTGAGGG	ATTCCTGCAT	GCAGAAAATC	GAAACCTAAC	6180
CGGATGCTCA	CACCCGTCAC	TACTGTTCTC	CTTCAATGCG	ACAGAAGCGT	TAACGAAAGA	6240
TGCTGGCAAC	AACACCTCGT	TATCCAGCCT	GGGATGGCCG	AGTTCCATCA	CCGATGATCT	6300
ACGCACGTTT	GGTGCTACCA	GCCAGAGTAT	GGGTGCTCTC	TACTGTATTG	GGATAGGATT	6360
AGCGGGACTG	GCGGTTTTTG	AACGATTGTG	GTTCTGTGATC	GCGAAAGGGC	CGAGACAGAC	6420
GGTTGTAGAA	GTTTCTTCTC	TTATGGTAGG	CTCCATTGAA	CGTTCTAGAT	CCCGTCCGGT	6480
ACTAAGAAGG	CCCACAGCTC	AGTTTCACTA	TGCTCAGCAT	ACCGTCTATC	ATCGCAACCG	6540
TCGTTGCCTT	ACAATTTGTG	AGCCTCATCA	ATCGTCACGG	AGAGGAGTCT	GGTGTGACAG	6600
CGAGATATGG	ACATCAATTT	TTAGGAATGA	CGTGGGCAGC	CGTCGGGTTG	TTGCTGGTCG	6660
GAAGCACTGT	CAGTTTACTG	ACGGTATTGG	TGGACCGCAA	CCGATCGGCA	GACCAGTATG	6720
AACCGGTGGC	AGAACCGAAG	ACGGTGGCCG	AGGATTCCGA	CTCGGTAGCG	TCGAACCAGA	6780
AGGGGGACTA	AGAAAGGAAC					6800

(2) INFORMATION FOR SEQ ID NO:26:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 391 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:26:

Met	Gly	Lys	Lys	Ala	Ile	Gln	Phe	Gly	Gly	Gly	Asn	Ile	Gly	Arg	Gly	
1				5				10						15		
Phe	Val	Ala	Glu	Phe	Leu	His	Ala	Ala	Gly	Tyr	Glu	Val	Val	Phe	Ile	
		20						25						30		
Asp	Val	Met	Asp	Ser	Val	Ile	Asn	Ser	Leu	Gln	Gln	Thr	Pro	Ser	Tyr	
		35					40						45			
Asp	Val	Thr	Glu	Val	Ser	Glu	Glu	Gly	Glu	Ser	Thr	Lys	Thr	Ile	Thr	
		50				55					60					
Asn	Tyr	Arg	Ala	Ile	Asn	Ser	Lys	Thr	His	Glu	Ala	Asp	Val	Val	Gln	
		65			70					75						
Glu	Ile	Ala	Ser	Ala	Asp	Val	Val	Thr	Cys	Ala	Val	Gly	Pro	Asn	Ile	
			85						90				95			
Leu	Lys	Phe	Ile	Ala	Pro	Val	Ile	Ala	Lys	Gly	Ile	Asp	Ala	Arg	Thr	
		100						105					110			
Glu	Glu	Arg	Pro	Val	Ala	Val	Ile	Ala	Cys	Glu	Asn	Ala	Ile	Gly	Ala	
		115					120					125				
Thr	Asp	Thr	Leu	His	Gly	Tyr	Ile	Lys	Gln	His	Thr	Asn	Pro	Asp	Arg	
		130			135						140					
Leu	Glu	Thr	Leu	Ser	Glu	Arg	Ala	Arg	Phe	Ala	Asn	Ser	Ala	Ile	Asp	
		145			150					155						
Arg	Ile	Val	Pro	Asn	Gln	Pro	Pro	Asn	Ser	Gly	Leu	Asn	Val	Arg	Ile	
			165					170						175		
Glu	Lys	Phe	Tyr	Glu	Trp	Ala	Val	Glu	Lys	Thr	Pro	Phe	Gly	Glu	Trp	
		180						185					190			
Gly	His	Pro	Asp	Ile	Pro	Ala	Ile	His	Trp	Val	Asp	His	Leu	Glu	Pro	
		195				200					205					
Tyr	Ile	Glu	Arg	Lys	Leu	Phe	Thr	Val	Asn	Thr	Gly	His	Ala	Thr	Thr	
		210				215					220					
Ala	Tyr	Tyr	Ala	His	Lys	Arg	Gly	Lys	Lys	Met	Ile	Ala	Glu	Ala	Leu	
		225			230					235						

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Glu Asp Pro Glu Ile Arg Glu Thr Val His Lys Val Leu Glu Glu Thr
                245                250                255
Ala Ser Leu Ile Val Ser Lys His Glu Ile Ser Glu Gln Glu Gln Lys
                260                265                270
Glu Tyr Val Asp Lys Ile Val Ser Arg Ile Ser Asn Pro Tyr Leu Glu
                275                280                285
Asp Asn Val Glu Arg Val Gly Arg Ala Pro Leu Arg Lys Leu Ser Arg
                290                295                300
Lys Glu Arg Phe Ile Gly Pro Ala Ser Gln Leu Ala Glu Arg Gly Gln
305                310                315                320
Lys Phe Asp Ala Leu Leu Gly Ala Ile Glu Met Ala Leu Arg Phe Gln
                325                330                335
Asn Val Pro Gly Asp Glu Glu Ser Ser Glu Leu Ala Arg Ile Leu Lys
                340                345                350
Glu Asn Ser Ala Glu Asp Ala Thr Ser Gln Leu Thr Gly Leu Glu Lys
                355                360                365
Asp His Pro Leu Tyr Ser His Val Val Glu Arg Val Ser Thr Val Gln
370                375                380
Gln Gly Ser Lys Ser Val Leu
385                390

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(2) INFORMATION FOR SEQ ID NO:27:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 195 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:27:

```

Met Val Asp Gln Ala Gln Asp Thr Leu Arg Pro Asn Asn Arg Leu Ser
 1                5                10                15
Asp Met Gln Ala Thr Met Glu Gln Thr Gln Ala Phe Glu Asn Arg Val
                20                25                30
Leu Glu Arg Leu Asn Ala Gly Lys Thr Val Arg Ser Phe Leu Ile Thr
                35                40                45
Ala Val Glu Leu Leu Thr Glu Ala Val Asn Leu Leu Val Leu Gln Val
                50                55                60
Phe Arg Lys Asp Asp Tyr Ala Val Lys Tyr Ala Val Glu Pro Leu Leu
65                70                75                80
Asp Gly Asp Gly Pro Leu Gly Asp Leu Ser Val Arg Leu Lys Leu Ile
                85                90                95
Tyr Gly Leu Gly Val Ile Asn Arg Gln Glu Tyr Glu Asp Ala Glu Leu
                100                105                110
Leu Met Ala Leu Arg Glu Glu Leu Asn His Asp Gly Asn Glu Tyr Ala
                115                120                125
Phe Thr Asp Asp Glu Ile Leu Gly Pro Phe Gly Glu Leu His Cys Val
130                135                140
Ala Ala Leu Pro Pro Pro Gln Phe Glu Pro Ala Asp Ser Ser Leu
145                150                155                160
Tyr Ala Met Gln Ile Gln Arg Tyr Gln Gln Ala Val Arg Ser Thr Met
                165                170                175
Val Leu Ser Leu Thr Glu Leu Ile Ser Lys Ile Ser Leu Lys Lys Ala
180                185                190
Phe Gln Lys
195

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(2) INFORMATION FOR SEQ ID NO:28:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 366 amino acids
- (B) TYPE: amino acid

(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEO ID NO:28:

Met 1	Ile	Ala	Leu	His 5	Phe	Gly	Ala	Gly	Asn 10	Ile	Gly	Arg	Gly	Phe 15	Ile
Gly	Ala	Leu	Leu 20	His	His	Ser	Gly	Tyr 25	Asp	Val	Val	Phe	Ala 30	Asp	Val
Asn	Glu	Thr 35	Met	Val	Ser	Leu	Leu 40	Asn	Glu	Lys	Lys	Glu 45	Tyr	Thr	Val
Glu	Leu 50	Ala	Glu	Glu	Gly	Arg 55	Ser	Ser	Glu	Ile	Ile 60	Gly	Pro	Val	Ser
Ala 65	Ile	Asn	Ser	Gly 70	Ser	Gln	Thr	Glu	Glu 75	Leu	Tyr	Arg	Leu	Met	Asn 80
Glu	Ala	Ala	Leu	Ile 85	Thr	Thr	Ala	Val 90	Gly	Pro	Asn	Val	Leu 95	Lys	Leu
Ile	Ala	Pro	Ser 100	Ile	Ala	Glu	Gly	Leu 105	Arg	Arg	Arg	Asn	Thr 110	Ala	Asn
Thr	Leu	Asn 115	Ile	Ile	Ala	Cys	Glu 120	Asn	Met	Ile	Gly	Gly 125	Ser	Ser	Phe
Leu	Lys 130	Lys	Glu	Ile	Tyr	Ser	His 135	Leu	Thr	Glu	Ala 140	Glu	Gln	Lys	Ser
Val 145	Ser	Glu	Thr	Leu	Gly 150	Phe	Pro	Asn	Ser	Ala 155	Val	Asp	Arg	Ile	Val 160
Pro	Ile	Gln	His 165	His	Glu	Asp	Pro	Leu 170	Lys	Val	Ser	Val	Glu 175	Pro	Phe
Phe	Glu	Trp 180	Val	Ile	Asp	Glu	Ser	Gly 185	Phe	Lys	Gly	Lys	Thr 190	Pro	Val
Ile	Asn 195	Gly	Ala	Leu	Phe	Val	Asp 200	Asp	Leu	Thr	Pro 205	Tyr	Ile	Glu	Arg
Lys	Leu 210	Phe	Thr	Val	Asn 215	Thr	Gly	His	Ala	Val	Thr 220	Ala	Tyr	Val	Gly
Tyr 225	Gln	Arg	Gly	Leu	Lys 230	Thr	Val	Lys	Glu	Ala 235	Ile	Asp	His	Pro	Glu 240
Ile	Arg	Arg	Val	Val	His 245	Ser	Ala	Leu 250	Leu	Glu	Thr	Gly	Asp 255	Tyr	Leu
Val	Lys	Ser 260	Tyr	Gly	Phe	Lys	Gln	Thr 265	Glu	His	Glu	Gln	Tyr 270	Ile	Lys
Asn	Gln 275	Arg	Ser	Leu	Leu	Lys	Ser 280	Phe	His	Phe	Gly	Arg 285	Cys	Asp	Pro
Arg	Ser 290	Glu	Val	Thr	Ser	Gln	Lys 295	Thr	Gly	Arg	Lys 300	Cys	Arg	Leu	Val
Gly 305	Pro	Ala	Lys	Lys	Ile 310	Lys	Glu	Pro	Asn 315	Ala	Leu	Ala	Glu	Gly	Ile 320
Ala	Ala	Ala	Leu	Arg 325	Phe	Asp	Phe	Thr	Gly 330	Asp	Pro	Glu	Ala 335	Val	Glu
Leu	Gln	Ala 340	Leu	Ile	Glu	Glu	Lys	Asp 345	Thr	Ala	Ala	Tyr	Phe 350	Lys	Arg
Cys	Ala	Ala 355	Phe	Ser	Pro	Met	Asn 360	Arg	Cys	Thr	Pro	Ser 365	Phe		

(2) INFORMATION FOR SEQ ID NO:29:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 3300 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:29:

GGATTGGCTG ACTGCGTGTT GTTACTCCAG GGATTGCGAC GAGCACTTCC TAAGCCACCG 60
TGCACGGAG GCGACTCTGA GCGTGACGAT GCCCTAGCGA ACCCGCGGAA GCCCATCGTG 120

GGGCGAAAT	CTTCTTCGTC	GGAGCTCATG	TCGGCGATTT	TACGTTTTTG	GCCGGCGGAT	180
GGTGAGGCCG	GGGGAGAATC	CGAATCCATG	ACCGATCGCA	GATGTCAGGA	TAAGGTGTAA	240
GTAGTAATC	AGAGTCGTCT	GAGAGGTTCT	AAAGGCAATG	AAACGGTCAA	ACGACACGTT	300
TGAGAGCCAC	GAAGGAGCTG	TGGGTTGAGA	TACGCACGAT	AACGAGAAAG	GAAAGTTGAT	360
TATCGGACAT	TTCGGGCGCG	GGAAAATTCA	AGTCCGAGGG	GCCGAGCAAC	AATGACGTTT	420
GTTGCATCGA	ATCTCCCTTC	CGGTTATTTT	TCCCTCTTCT	TCTCCTCTTC	TTCTTTTCTT	480
CTTTACCCTC	TCCTCTCTTT	GGCATTTCGT	CACTACTTTG	TAACGTAACT	CAATTCTATT	540
GATACATAAA	AATCACATAT	CAACTATGGC	TGCCTCTCTT	ATCCGTACCT	CTGCCCCTAC	600
CGCTCTTCGC	GCTGGAGCTT	CGGCTACTCC	TAAAGCTGCG	GGTGTGCGG	GTTTGACCTT	660
TGCCCCGTGG	AAGGCCACTC	TGCCTGACCT	GGCTTGTATG	GCTCTCCCTT	TCCCTTGATG	720
TCGTCAATTT	GCCCCCTCTG	TGTGTTATCT	TCCGTTTTGT	CATCTTTCTC	GGCTATTTTG	780
GCAGTGCGAA	TGAGTAGATG	GGTTACGCTT	GTCGCTCATG	ACGCCCCGGA	AGCACGTAAT	840
GCAATGGTTG	GTTGACTGAA	TAACAGATGA	CTATGGCGCC	CTTGAGCCCT	CTATCTCCGG	900
AAAGATCATG	GAGCTTCACC	ACAAGAACCA	CCACCAGACC	TATGTCAACA	GCTACAACAC	960
CGCCATCGAA	CAGCTCCAGG	AGGCCGTCGC	CAAGGAGGAC	ATCACCACCT	AGATCAACCT	1020
CAAGCCCCCTG	ATCAACTTCC	ACGGTGGTGG	CCACATCAAC	CACACTCTTT	TCTGGGAGAA	1080
CCTTGCCCCCT	AAGAGCCAGG	GCGGTGGTGA	GCCCCATCT	GGAGCTTTGG	CCAAGGCCAT	1140
CGACGAAAGC	TTCCGGCAGCT	TGGGAGAGTT	CCAGAGCAAG	ATGAACGCCG	CCCTCGCTGG	1200
TATTCAGGGA	AGCGGATGGG	CTTGGCTCGT	CAAGGACAAG	CAGACCGGAA	ACATCGGCAT	1260
CAAGACCTAT	GCCGTAAGTT	CCTCCTTGTT	AGCGCCTAAG	GATACAGGTA	GCTAACTCCC	1320
GACCAGAACC	AGGACCCTGT	CGTTGGTCAG	TTCCAGCCTC	TTCTCGGTAT	TGATGCTTGG	1380
GAGCAGCCT	ACTAGTAAGT	TTTCTTGAGC	TAGATATCTA	CCAAGCAATA	ACTAATGCCG	1440
TGTTAGCCTT	CAATACCAGA	ACCGCAAGGC	TGAGTACTTC	AGCGCCATCT	GGGACGTCAT	1500
CAACTGGAAG	GCGGTTGAGA	AGCGCTTCTC	GTAAGCGTGC	AAAAGTGTTG	TGAATTGACG	1560
CAGCTTGATG	AGCGCTTTGT	TTCAAGTTGTG	CCAGAGTGA	TACTGTGTAA	TGTCTGATCA	1620
AGCTGTACTT	GTAGCCCTAA	TGCAATTGGA	TACGCCTCGT	GTATATATAA	ACTCATGTTT	1680
GTGAACGTA	AATAATTTTG	GGGAAGCTGC	ACCAGCCACA	GTGGCTGGAT	CACATGCTCC	1740
CGTAGCATTC	CCGCAGTTTC	CGGCAAGCTT	ATTTTCTTAG	TTTGGGATCC	GCTCCGCCCT	1800
CTCCGGATCT	TCTTCCCTCA	TCTCACCTCT	CAAGCGATCA	ATTCTCTCGA	AATGTCTGCA	1860
GAAAGCCCCG	GAGAAAAGCG	CGGTGGGTTT	CGGGCGTTCT	TCGCCGGCGC	CCTCCGACCT	1920
AAGAAATCCC	GTCAGGTCCT	CCGAAAGGCA	TCGACACCGA	ATCTAAAGGA	AGGTCTACAA	1980
AGCAAAGATG	ACGTCCCCGC	GATGCCTTCA	CTGACCCCAT	TGGAGGCCCA	CCGACTCAAA	2040
TACCGAGAAG	TAAATCTTCA	GAAAGACACA	CAGCTAGGCG	AAACCCACGA	TCATACCGCA	2100
ATGCTGCATT	CAATCGGTGT	TGGAGAGCTC	GATCCGTCGG	ATCCACACGC	GCAACTACAC	2160
GAATTGACA	ATAGACCCCC	AGGCGAGCCT	ATGATTGCGA	GCTTAACATC	GGACCTCTGG	2220
GCCAAGGTCA	CCGAGTATCT	CAATCCCCGC	GAAAGAGCCA	GTCTTGCTTT	CTCCAGCCGA	2280
ACACTATACG	CTCGTCTGGG	CCGCGAGCCC	TGGATAACAA	TAAACCTCCC	AGAAAACCAC	2340
GACTACAAAG	CGGACTTTCT	CATCTCCCAA	GATAGACTAC	TCCCTCACCA	TCTCCTCTGT	2400
TTCCCCTGCG	GCAAATACCA	CCGCCGCACA	CAAGAAGGCT	ACGAAAAGCT	CCAACCCGCA	2460
GACATAATCA	ACCCGCTCTT	CGATTGCCCC	AACGCCCGCA	ACAACGCCCT	CCCAGCACCC	2520
CGCCACCGCA	TCACCCACGG	CCGAGTCCTT	TACTTCACCT	TCCACCAGCT	AGTCATGCGC	2580
GCATACCGAT	TTGGACCCCG	CTACGGCATC	TCAGCCGACT	CTCTATCCCG	TCGCTGGCGC	2640
CGGGACGGCT	GGTCCCACCA	AACCCGATAC	CACATCCATC	AGGGTCGACT	GCTCATGCGA	2700
GTCGTGAGCA	CCTGCTTCGC	CGAACCAGGC	CTCAGCGCCA	GCCAACAGCG	ACTCCTCCTC	2760
TACTCGCGCG	ACGACTACTG	GCCGTACTTC	TCCGTCTGCG	CGCACTGGCG	GGATGGCGAA	2820
CTTATGAACG	TTTGCAAATG	CGCCCTCGGC	CACATCCCCG	TCCCCCGCAC	CACGAACGGC	2880
CTGCAGGGCC	TGCAACACCG	CGCAAAAGAT	ATGTACCACC	GTCGAGAGCA	CAATCCCCAAC	2940
GCCCTCGCGT	CGCTCTGCGG	TAAGTGTGCA	CCTATGCGTC	GCTGCCCCGA	GTGTCCCTCC	3000
GAGTATCTGG	TCGAGGTCAA	GCTCACCGAG	GACCGGAGTG	GTTGCGATCG	CAACTTATTC	3060
CGGCATGCGA	TTGTGGTGAC	ACGGTGGAGT	GATTTGGGGG	ATGGGCGGTC	GCCGCGGCTA	3120
TCGAAGGAGT	GGGCGGCGAT	TAATGGGGAC	GAGGCGGGTG	AGGGGTATGA	TTCTTTTGAG	3180
AAAATAGGGA	AGAGGGCTAT	TTCCGGGGATT	TTTGAGTCGG	CTATTACCGA	TGATACTTTG	3240
CCTGGGCAGA	GGATTCTTTC	AATGAATCCT	AAGGGAAAGA	AGTTGGGTGA	GGCTGGGAAT	3300

(2) INFORMATION FOR SEQ ID NO:30:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 230 amino acids
 - (B) TYPE: amino acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:30:

```

Met Ala Ala Ser Leu Ile Arg Thr Ser Ala Arg Thr Ala Leu Arg Ala
 1          5          10          15
Gly Ala Ser Ala Thr Pro Lys Ala Ala Gly Val Ala Gly Leu Thr Phe
          20          25          30
Ala Arg Gly Lys Ala Thr Leu Pro Asp Leu Ala Tyr Asp Tyr Gly Ala
          35          40          45
Leu Glu Pro Ser Ile Ser Gly Lys Ile Met Glu Leu His His Lys Asn
          50          55          60
His His Gln Thr Tyr Val Asn Ser Tyr Asn Thr Ala Ile Glu Gln Leu
65          70          75          80
Gln Glu Ala Val Ala Lys Glu Asp Ile Thr Thr Gln Ile Asn Leu Lys
          85          90          95
Pro Leu Ile Asn Phe His Gly Gly Gly His Ile Asn His Thr Leu Phe
          100          105          110
Trp Glu Asn Leu Ala Pro Lys Ser Gln Gly Gly Gly Glu Pro Pro Ser
          115          120          125
Gly Ala Leu Ala Lys Ala Ile Asp Glu Ser Phe Gly Ser Leu Gly Glu
130          135          140
Phe Gln Ser Lys Met Asn Ala Ala Leu Ala Gly Ile Gln Gly Ser Gly
145          150          155          160
Trp Ala Trp Leu Val Lys Asp Lys Gln Thr Gly Asn Ile Gly Ile Lys
          165          170          175
Thr Tyr Ala Asn Gln Asp Pro Val Val Gly Gln Phe Gln Pro Leu Leu
          180          185          190
Gly Ile Asp Ala Trp Glu His Ala Tyr Tyr Leu Gln Tyr Gln Asn Arg
          195          200          205
Lys Ala Glu Tyr Phe Ser Ala Ile Trp Asp Val Ile Asn Trp Lys Ala
210          215          220
Val Glu Lys Arg Phe Ser
225          230

```

(2) INFORMATION FOR SEQ ID NO:31:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 233 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:31:

```

Met Phe Ala Lys Thr Ala Ala Ala Asn Leu Thr Lys Lys Gly Gly Leu
 1          5          10          15
Ser Leu Leu Ser Thr Thr Ala Arg Arg Thr Lys Val Thr Leu Pro Asp
          20          25          30
Leu Lys Trp Asp Phe Gly Ala Leu Glu Pro Tyr Ile Ser Gly Gln Ile
          35          40          45
Asn Glu Leu His Tyr Thr Lys His His Gln Thr Tyr Val Asn Gly Phe
          50          55          60
Asn Thr Ala Val Asp Gln Phe Gln Glu Leu Ser Asp Leu Leu Ala Lys
65          70          75          80
Glu Pro Ser Pro Ala Asn Ala Arg Lys Met Ile Ala Ile Gln Gln Asn
          85          90          95
Ile Lys Phe His Gly Gly Gly Phe Thr Asn His Cys Leu Phe Trp Glu
          100          105          110
Asn Leu Ala Pro Glu Ser Gln Gly Gly Gly Glu Pro Pro Thr Gly Ala
          115          120          125
Leu Ala Lys Ala Ile Asp Glu Gln Phe Gly Ser Leu Asp Glu Leu Ile
130          135          140
Lys Leu Thr Asn Thr Lys Leu Ala Gly Val Gln Gly Ser Gly Trp Ala
145          150          155          160
Phe Ile Val Lys Asn Leu Ser Asn Gly Gly Lys Leu Asp Val Val Gln
          165          170          175

```

```

Thr Tyr Asn Gln Asp Thr Val Thr Gly Pro Leu Val Pro Leu Val Ala
      180      185      190
Ile Asp Ala Trp Glu His Ala Tyr Tyr Leu Gln Tyr Gln Asn Lys Lys
      195      200      205
Ala Asp Tyr Phe Lys Ala Ile Trp Asn Val Val Asn Trp Lys Glu Ala
      210      215      220
Ser Arg Arg Phe Asp Ala Gly Lys Ile
225      230

```

(2) INFORMATION FOR SEQ ID NO:32:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:32:

GCTCTAGATC GTCGGAGCTC ATGTCGGCGA TTTTAC 36

(2) INFORMATION FOR SEQ ID NO:33:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 36 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:33:

GCGGTACCAC GCCTAGAGCA AAGTATAAAT AAGGAA 36

(2) INFORMATION FOR SEQ ID NO:34:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 346 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:34:

```

ATATCGACTG TAGTAATATC TCAGGTCTCT GGGATAGCTG ATGGAATGTT AAGTGAATAA 60
TATTGATTTA AAGTTCCTCT AGTTCCAAGC TCTTATGTAG CTTTATTTTC TATATATATA 120
TATTCTATTT AGTGGTGTG CAGGCGGTGA GCCTATCGGC CAATCATAGT AAAAAACCCG 180
TTAGTTGCAA TACCCTGTGA GTTGCAAGGC GAATTCCTGG CTGATATCCT TGCAACTAAC 240
GGGGTTTCTC AGTACTCGAA TTGAATATAT ATTTGCGACA AAGTTATTTT GCAAACCTTG 300
GGGCCCTGGG GTCATACAAC CCAAGCCACA AGCTTTATTT AATTCG 346

```

(2) INFORMATION FOR SEQ ID NO:35:

- (i) SEQUENCE CHARACTERISTICS:
- (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:35:

CTATGATTGG CCGATAGG 18

(2) INFORMATION FOR SEQ ID NO:36:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:36:

CCAGGCTCGC ACGCTTTC 18

(2) INFORMATION FOR SEQ ID NO:37:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:37:

CTTGCAACTA ACGGGGTT 18

(2) INFORMATION FOR SEQ ID NO:38:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:38:

TGAGAAAGAC CAAGAATG 18

(2) INFORMATION FOR SEQ ID NO:39:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 188 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:39:

GATTTAATGA CTACCTTGAT GATACTGCCA ATATAGTTAG ATAATACAAA TCCTGGCTGC	60
CATATAACGC CCTCGCAAAC GACATCTTGT TCTTATTNTC CCTCAATCGA GCTTGCCTAT	120
GCCCAAGCTT CGAACTATAC GAGCATTGTA AATTGATTTT GATACGGCCT GCCATATCAG	180
ATTGACTC	188

(2) INFORMATION FOR SEQ ID NO:40:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:40:

TGTAGTCTGA CTAGCATG 18

(2) INFORMATION FOR SEQ ID NO:41:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:41:

GGATCTTCAC CTAGATCC

18

(2) INFORMATION FOR SEQ ID NO:42:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:42:

CATAGTGTCTG ACCAAGC

17

(2) INFORMATION FOR SEQ ID NO:43:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:43:

CAATCGAGCT TGCCTATG

18

(2) INFORMATION FOR SEQ ID NO:44:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 27 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:44:

ATTTAAATGG TCCTCGGTGG ATCAAGC

27

(2) INFORMATION FOR SEQ ID NO:45:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 28 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:45:

TTAATTAATT AGTCCTGTCT GCGCTGGT

28

(2) INFORMATION FOR SEQ ID NO:46:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 39 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:46:

CGGTGGATCA AGCGGTTAAT TAATCACTCC GTACCTGAT

39

(2) INFORMATION FOR SEQ ID NO:47:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 17 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:47:

GCACTCGAAT GACTACT

17

(2) INFORMATION FOR SEQ ID NO:48:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:48:

CGCATCATAC TTGCGACA

18

(2) INFORMATION FOR SEQ ID NO:49:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 21 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:49:

AGAAATCGGG TATCCTTTCA G

21

(2) INFORMATION FOR SEQ ID NO:50:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 1132 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:50:

AACCCTGTAT	GTGAGCCTGA	TTCAAGACTT	CGGATTTACT	TTCAAGGCTT	CGAATCACTG	60
TTAAGGCAGA	AGAAAGTAGT	ACTAATGGTT	ATCAATATAT	AGGAGGGCAA	GCGGGAGGTT	120
ATCGCCAACG	AAGAAGGAGG	TTAGTCCACT	ACTGCTTGGT	GGCGGGTAAT	CTTAAGAGCA	180
CAAACTAACG	GATACACAGA	TCGTCAAATC	CCTTCCGTCC	TTTCATACAT	TGATGGTGAG	240
GAGTACCACG	GTACTCAAGC	CAAGGCCAG	TTGGTCCGCA	ACTCCCAGAA	CACTGTCGCA	300

TACTTCAGAG	ATTACCTTGG	CAAGGAGTTC	AAGTCGATAG	ACGCCACACC	ATGCCATAAC	360
TCGGCGCATC	CTCAGCCTCA	CGAGTCTACC	GTTGCTTTCT	CCATTGTGGA	CTCTACCAAC	420
GAGACCCCA	GCACTGTCAC	CGTCTCCGAG	ATTGCCACCC	GCCATCTCCG	TCGTTTGAAG	480
CAGTCCGCT	CTGACTACCT	GGGCAAGGAA	GTCAATGCCG	CCGTCATCAC	TGTCCCCACT	540
GACTTCTCCG	ATGCTCAGCG	CGAGGCTTTG	ACCGCTTCCG	CTAAGGCTGC	TGGCCTTGAG	600
GTCCTCCAG	TCATCCATGA	GCCTGTTGCC	GCTGCCCTGG	CTTACGATGC	CAGGCCGAG	660
GCTACTGTTA	CTGACAAGCT	TGTTGTCGTC	GCCGACCTCG	GTGGTACCCG	ATCCGACGCT	720
GCTGTTCTCG	CTTGCCGTGG	TGGCATGTAC	AGTATCCTCG	CAACTGCTCA	TGACTACGAG	780
TTGGGTGGAG	CTTCGTTGGA	CAAGATCATC	ATCGACCATT	TCGCCAAGGA	GTTCATTAAG	840
AAGCACAAGA	CCGATCCTCG	CGAGAACGCT	CGTGGTCTCG	CCAAGTTGAA	GCTTGAGGGT	900
GAGGCTGCTC	GCAAGACCTT	GAGCTTGGGT	ACCAACGCCA	GCTTGAGCAT	TGAGATCTTC	960
GCAGATGGCA	TTGATTTTCG	CTCCACTGTC	AACCGTACTC	GNTACGAAC	TCTTTCCGGC	1020
AAGACCTTCG	CCCAGTTCAC	CGGCTTGATC	GAGCAGGTTA	TCCAGAAGGC	TGGTTTGGAT	1080
GTTTTGGACA	TTGACGAGGT	TAGTCCCTTG	TGATTTTTTT	TTTTTTTTTC	AG	1132

(2) INFORMATION FOR SEQ ID NO:51:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 374 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:51:

Asn	Pro	Val	Cys	Glu	Pro	Asp	Ser	Arg	Leu	Arg	Ile	Tyr	Phe	Gln	Gly	
1				5				10						15		
Phe	Glu	Ser	Leu	Leu	Arg	Gln	Lys	Lys	Val	Val	Leu	Met	Val	Ile	Asn	
			20					25					30			
Ile	Glu	Gly	Lys	Arg	Glu	Val	Ile	Ala	Asn	Glu	Glu	Gly	Gly	Ser	Thr	
		35					40					45				
Thr	Ala	Trp	Trp	Arg	Val	Ile	Leu	Arg	Ala	Gln	Thr	Asn	Gly	Tyr	Thr	
	50					55					60					
Asp	Arg	Gln	Ile	Pro	Ser	Val	Leu	Ser	Tyr	Ile	Asp	Gly	Glu	Glu	Tyr	
65				70					75					80		
His	Gly	Thr	Gln	Ala	Lys	Ala	Gln	Leu	Val	Arg	Asn	Ser	Gln	Asn	Thr	
			85					90						95		
Val	Ala	Tyr	Phe	Arg	Asp	Tyr	Leu	Gly	Lys	Glu	Phe	Lys	Ser	Ile	Asp	
		100						105					110			
Ala	Thr	Pro	Cys	His	Asn	Ser	Ala	His	Pro	Gln	Pro	His	Glu	Ser	Thr	
		115					120					125				
Val	Ala	Phe	Ser	Ile	Val	Asp	Ser	Thr	Asn	Glu	Thr	Pro	Ser	Thr	Val	
	130					135					140					
Thr	Val	Ser	Glu	Ile	Ala	Thr	Arg	His	Leu	Arg	Leu	Lys	Gln	Ser		
145				150					155					160		
Ala	Ser	Asp	Tyr	Leu	Gly	Lys	Glu	Val	Asn	Ala	Ala	Val	Ile	Thr	Val	
			165					170					175			
Pro	Thr	Asp	Phe	Ser	Asp	Ala	Gln	Arg	Glu	Ala	Leu	Thr	Ala	Ser	Ala	
		180					185						190			
Lys	Ala	Ala	Gly	Leu	Glu	Val	Leu	Gln	Leu	Ile	His	Glu	Pro	Val	Ala	
	195						200					205				
Ala	Ala	Leu	Ala	Tyr	Asp	Ala	Arg	Pro	Glu	Ala	Thr	Val	Thr	Asp	Lys	
	210					215					220					
Leu	Val	Val	Val	Ala	Asp	Leu	Gly	Gly	Thr	Arg	Ser	Asp	Ala	Ala	Val	
225				230					235					240		
Leu	Ala	Cys	Arg	Gly	Gly	Met	Tyr	Ser	Ile	Leu	Ala	Thr	Ala	His	Asp	
			245					250						255		
Tyr	Glu	Leu	Gly	Gly	Ala	Ser	Leu	Asp	Lys	Ile	Ile	Ile	Asp	His	Phe	
	260						265						270			
Ala	Lys	Glu	Phe	Ile	Lys	Lys	His	Lys	Thr	Asp	Pro	Arg	Glu	Asn	Ala	
	275						280					285				
Arg	Gly	Leu	Ala	Lys	Leu	Lys	Leu	Glu	Gly	Glu	Ala	Ala	Arg	Lys	Thr	
	290					295					300					

```

Leu Ser Leu Gly Thr Asn Ala Ser Leu Ser Ile Glu Ile Phe Ala Asp
305                               310                               320
Gly Ile Asp Phe Gly Ser Thr Val Asn Arg Thr Arg Tyr Glu Leu Leu
                               325                               330                               335
Ser Gly Lys Thr Phe Ala Gln Phe Thr Gly Leu Ile Glu Gln Val Ile
                               340                               345                               350
Gln Lys Ala Gly Leu Asp Val Leu Asp Ile Asp Glu Val Ser Pro Leu
                               355                               360                               365
Phe Phe Phe Phe Ser
                               370

```

(2) INFORMATION FOR SEQ ID NO:52:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 339 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:52:

```

Met Ser Lys Ala Val Gly Ile Asp Leu Gly Thr Thr Tyr Ser Cys Val
1                               5                               10                               15
Ala His Phe Ala Asn Asp Arg Val Asp Ile Ile Ala Asn Asp Gln Gly
                               20                               25                               30
Asn Arg Thr Thr Pro Ser Phe Val Ala Phe Thr Asp Thr Glu Arg Leu
                               35                               40                               45
Ile Gly Asp Ala Ala Lys Asn Gln Ala Ala Met Asn Pro Ser Asn Thr
                               50                               55                               60
Val Phe Asp Ala Lys Arg Leu Ile Gly Arg Asn Phe Asn Asp Pro Glu
65                               70                               75                               80
Val Gln Ala Asp Met Lys His Phe Pro Phe Lys Leu Ile Asp Val Asp
                               85                               90                               95
Gly Lys Pro Gln Ile Gln Val Glu Phe Lys Gly Glu Thr Lys Asn Phe
                               100                              105                              110
Thr Pro Glu Gln Ile Ser Ser Met Val Leu Gly Lys Met Lys Glu Thr
                               115                              120                              125
Ala Glu Ser Tyr Leu Gly Ala Lys Val Asn Asp Ala Val Val Thr Val
                               130                              135                              140
Pro Ala Tyr Phe Asn Asp Ser Gln Arg Gln Ala Thr Lys Asp Ala Gly
145                              150                              155                              160
Thr Ile Ala Gly Leu Asn Val Leu Arg Ile Ile Asn Glu Pro Thr Ala
                               165                              170                              175
Ala Ala Ile Ala Tyr Gly Leu Asp Lys Lys Gly Lys Glu Glu His Val
                               180                              185                              190
Leu Ile Phe Asp Leu Gly Gly Gly Thr Phe Asp Val Ser Leu Leu Phe
                               195                              200                              205
Ile Glu Asp Gly Ile Phe Glu Val Lys Ala Thr Ala Gly Asp Thr His
                               210                              215                              220
Leu Gly Gly Glu Asp Phe Asp Asn Arg Leu Val Asn His Phe Ile Gln
225                              230                              235                              240
Glu Phe Lys Arg Lys Asn Lys Lys Asp Leu Ser Thr Asn Gln Arg Ala
                               245                              250                              255
Leu Arg Arg Leu Arg Thr Ala Cys Glu Ser Gln Glu Asn Phe Val Ser
                               260                              265                              270
Ser Ala Gln Thr Ser Val Glu Ile Asp Ser Lys Asn Glu Gly Ile Asp
                               275                              280                              285
Phe Tyr Thr Ser Ile Thr Arg Ala Arg Phe Glu Glu Leu Cys Ala Asp
                               290                              295                              300
Leu Phe Arg Ser Thr Leu Asp Pro Val Glu Lys Val Leu Arg Asp Ala
305                              310                              315                              320
Lys Leu Asp Lys Ser Gln Val Asp Glu Ile Val Leu Val Gly Gly Ser
                               325                              330                              335
Thr Arg Ile

```

(2) INFORMATION FOR SEQ ID NO:53:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 560 amino acids
 (B) TYPE: amino acid
 (C) STRANDEDNESS: single
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:53:

```

Lys Thr Leu Pro Phe Ser Glu Asn Phe Ile Met Ala Asp Ser Glu Glu
 1          5          10          15
Tyr Lys Thr Val Ile Gly Ile Ser Phe Gly Asn Gln Asn Ser Ser Ile
          20          25          30
Ala Phe Asn Arg Asp Gly Lys Thr Asp Val Leu Ala Asn Glu Glu Gly
          35          40          45
Asn Arg Gln Ile Pro Ser Ile Leu Pro Tyr His Gly Asp Gln Glu Tyr
          50          55          60
His Gly Val Gln Ala Arg Gly Gln Leu Val Arg Asn Ala Asp Asn Ser
          65          70          75          80
Val Thr Asn Phe Arg Asp Leu Leu Gly Lys Ser His Asp Glu Leu Thr
          85          90          95
His His His Cys His Tyr Ser Ser Asn Pro Val Asn Val Glu Gly Gln
          100          105          110
Ile Gly Phe Lys Ile Thr Val Gln Glu Gly Glu Glu Ser Asp Pro Lys
          115          120          125
Glu Lys Ile Leu Thr Ala His Glu Ala Ser Val Arg His Leu Arg Arg
          130          135          140
Leu Thr Glu Ser Ala Glu Asp Phe Leu Gly Thr Lys Val Asn Gly Cys
          145          150          155          160
Val Met Ser Val Pro Val Tyr Phe Thr Asp Ala Gln Arg Lys Ala Leu
          165          170          175
Glu Ser Ala Ala Asn Glu Ala Gly Leu Pro Val Leu Gln Leu Ile His
          180          185          190
Asp Pro Ala Ala Val Ile Leu Ala Leu Met Tyr Ser Glu Glu Val Leu
          195          200          205
Ile Asp Lys Thr Val Val Val Ala Asn Phe Gly Ala Thr Arg Ser Glu
          210          215          220
Val Ser Val Val Ser Val Lys Gly Gly Leu Met Thr Ile Leu Ala Ser
          225          230          235          240
Val His Asp Glu Asn Leu Gly Gly Glu Gln Leu Thr Asp Val Leu Val
          245          250          255
Asn Phe Phe Ala Lys Glu Phe Glu Lys Lys Asn Gly Ile Asp Pro Arg
          260          265          270
Lys Asn Ala Arg Ser Leu Val Lys Leu Arg Ala Gln Cys Glu Ile Thr
          275          280          285
Lys Arg Val Leu Ser Asn Gly Thr Thr Ala Ser Ala Ala Val Asp Ser
          290          295          300
Leu Ala Asp Gly Ile Asp Phe His Ser Ser Ile Asn Arg Leu Arg Tyr
          305          310          315          320
Asp Leu Ala Ala Ser Ala Thr Leu Asn Arg Met Ala Asp Leu Val Thr
          325          330          335
Glu Ala Val Glu Lys Ala Asn Met Glu Pro Phe Asp Ile Ser Glu Val
          340          345          350
Ile Leu Ala Gly Gly Ala Ser Asn Thr Pro Lys Leu Thr Ser Leu Met
          355          360          365
Glu Ser Ile Phe Pro Glu Gln Thr Ile Ile Arg Ser Ser Ser Ser Val
          370          375          380
Thr Pro Leu Gln Leu Asp Pro Ser Glu Leu Thr Ala Ile Gly Ser Gly
          385          390          395          400
Val Gln Ala Ser Leu Ile Gly His Phe Asp Ala Ala Asp Ile Ala Ala
          405          410          415
Ser Thr Asp Ala Gln Val Val Asp Val Pro His Leu Thr Ala Pro Ile
          420          425          430

```

Gly	Ile	Asn	Glu	Gly	Glu	Asn	Phe	Val	Thr	Ile	Phe	Asp	Ile	Glu	Thr
		435					440					445			
Ala	Leu	Pro	Ala	Arg	Lys	Thr	Val	Glu	Val	Ile	Ala	Pro	Lys	Glu	Gly
	450					455					460				
Ala	Ala	Phe	Ile	Pro	Ile	Tyr	Glu	Ala	Glu	Arg	Ser	Val	Lys	Val	Thr
465					470					475				480	
Lys	Val	Glu	Pro	Glu	Pro	Ile	Asp	Glu	Glu	Ala	Phe	Ser	Asp	Asp	
			485					490				495			
Glu	Glu	Glu	Glu	Pro	Glu	Glu	Ile	Lys	Glu	Arg	Ile	Ala	Ile	Pro	Lys
		500						505				510			
Thr	Leu	Ile	Ala	Thr	Ile	Thr	Leu	Pro	Asp	Val	Ser	Pro	Asn	Ala	Lys
	515					520					525				
Ile	Glu	Leu	Val	Leu	Gln	Ile	Asp	Ala	Glu	Gly	Lys	Leu	Thr	Ala	Ser
	530				535						540				
Ala	Arg	Pro	Lys	Asp	Gly	Lys	Gly	Thr	Asn	Val	Arg	Gly	Ser	Thr	Ala
545					550				555						560

(2) INFORMATION FOR SEQ ID NO:54:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:54:

TACGGTTGAC AGTGGAGC

18

(2) INFORMATION FOR SEQ ID NO:55:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:55:

CACTGACTTC TCCGATGC

18

(2) INFORMATION FOR SEQ ID NO:56:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 501 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:56:

ATTTCCCAGC	GTCGTCGTTG	GTTGAACGGT	TCTTTTGCGG	CCGGTCTCTA	TTCGCTCATG	60
CATTTTCGGTC	GGATGTACAA	GAGTGGACAT	AACATCATCC	GTATGTTCTT	CTTGACATT	120
CAGATGTTGT	ACAACGTTTT	CAACACTATC	CTTACATGGT	TCTCCCTGGC	ATCTTACTGG	180
TTGACCACCA	CCGTCATCAT	GGAATTGGTC	GGAACGCCCA	GTGAGAGCAA	CGGTAACAAA	240
GGATTCCCCT	TCGGTAAATC	GGCGACCCCT	ATTATCAACA	CAATTGTGAA	GTATGTCTAC	300
CTCGGATTGT	TGCTCTTGCA	GTTCACTTTC	GCTCTCGGTA	ACCGCCCCAA	GGGATCCCGC	360
TTCTCGTACC	TGACATCTTT	CGTCGTATTC	GGTATCATTC	AAATCTACGT	TGTCGTCGAC	420
GCTCTGTACT	TGGTGGTTTC	TGCATTCAAG	GTGTTGGCGA	ATTCCTCAAG	TCGTTCTTCT	480
CGTCTTCCGG	CGCCAGCGCC	A				501

(2) INFORMATION FOR SEQ ID NO:57:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 178 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:57:

```

Ile Ser Gln Arg Arg Arg Trp Leu Asn Gly Ser Phe Ala Ala Gly Leu
 1          5          10
Tyr Ser Leu Met His Phe Gly Arg Met Tyr Lys Ser Gly His Asn Ile
          20          25          30
Ile Arg Met Phe Phe Leu His Ile Gln Met Leu Tyr Asn Val Phe Asn
          35          40          45
Thr Ile Leu Thr Trp Phe Ser Leu Ala Ser Tyr Trp Leu Thr Thr Thr
          50          55          60
Val Ile Met Asp Leu Val Gly Thr Pro Ser Glu Ser Asn Gly Asn Lys
65          70          75          80
Gly Phe Pro Phe Gly Lys Ser Ala Thr Pro Ile Ile Asn Thr Ile Val
          85          90          95
Lys Tyr Val Tyr Leu Gly Leu Leu Leu Leu Gln Phe Ile Leu Ala Leu
          100          105          110
Gly Asn Arg Pro Lys Gly Ser Arg Phe Ser Tyr Leu Thr Ser Phe Val
          115          120          125
Val Phe Gly Ile Ile Gln Ile Tyr Val Val Val Asp Ala Leu Tyr Leu
          130          135          140
Val Val Arg Ala Phe Thr Asn Ser Asp Ala Ile Asp Phe Val Thr Asp
145          150          155          160
Gln Gly Val Gly Glu Phe Leu Lys Ser Phe Phe Ser Ser Ser Gly Ala
          165          170          175
Ser Ala

```

(2) INFORMATION FOR SEQ ID NO:58:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 916 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:58:

```

Met Ala Tyr His Gly Ser Gly Pro Gln Ser Pro Gly Glu His Thr Tyr
 1          5          10
Asp Asp Gly His Gln Leu Arg Asp Leu Ser His Ser Asn Thr Ser Tyr
          20          25          30
Glu Glu Glu Ala Ser His Gly Leu Leu Ser Ser Gln Gln Ser Pro Phe
          35          40          45
Ala Gly Pro Phe Asp Asp Pro His Gln Gln Arg Gly Leu Thr Ala Ser
          50          55          60
Pro Val Gln Arg Pro Thr Ser Gly Tyr Ser Leu Thr Glu Ser Tyr Ala
65          70          75          80
Pro Asp Ala Ala Tyr His Asp Pro Tyr Ser Ala Asn Gln Ser Val Tyr
          85          90          95
Ser Gly His Ser Glu Asn Pro Ala Ala Phe Gly Val Pro Gly Arg
          100          105          110
Val Ala Ser Pro Tyr Ala Arg Ser Glu Thr Ser Ser Thr Glu Ala Trp
          115          120          125

```

Arg	Gln	Arg	Gln	Ala	Gly	Ala	Arg	Arg	Gly	Gly	Asn	Gly	Leu	Arg	Arg
130						135					140				
Tyr	Ala	Thr	Arg	Lys	Val	Lys	Leu	Val	Gln	Gly	Ser	Val	Leu	Ser	Val
145					150					155					160
Asp	Tyr	Pro	Val	Pro	Ser	Ala	Ile	Gln	Asn	Ala	Ile	Gln	Ala	Lys	Tyr
				165					170					175	
Arg	Asn	Asp	Leu	Glu	Gly	Gly	Ser	Glu	Glu	Phe	Thr	His	Met	Arg	Tyr
			180					185					190		
Thr	Ala	Ala	Thr	Cys	Asp	Pro	Asn	Glu	Phe	Thr	Leu	His	Asn	Gly	Tyr
		195					200					205			
Asn	Leu	Arg	Pro	Ala	Met	Tyr	Asn	Arg	His	Thr	Glu	Leu	Leu	Ile	Ala
	210					215					220				
Ile	Thr	Tyr	Tyr	Asn	Glu	Asp	Lys	Thr	Leu	Thr	Ala	Arg	Thr	Leu	His
225					230					235					240
Gly	Val	Met	Gln	Asn	Ile	Arg	Asp	Ile	Val	Asn	Leu	Lys	Lys	Ser	Glu
				245					250					255	
Phe	Trp	Asn	Lys	Gly	Gly	Pro	Ala	Trp	Gln	Lys	Ile	Val	Val	Cys	Leu
			260					265					270		
Val	Phe	Asp	Gly	Ile	Asp	Pro	Cys	Asp	Lys	Asp	Thr	Leu	Asp	Val	Leu
		275					280					285			
Ala	Thr	Val	Gly	Ile	Tyr	Gln	Asp	Gly	Val	Met	Lys	Arg	Asp	Val	Asp
	290					295					300				
Gly	Lys	Glu	Thr	Val	Ala	His	Ile	Phe	Glu	Tyr	Thr	Thr	Gln	Leu	Ser
305					310					315					320
Val	Thr	Pro	Asn	Gln	Leu	Ile	Arg	Pro	Thr	Asp	Asp	Gly	Pro	Ser	
				325					330				335		
Thr	Leu	Pro	Pro	Val	Gln	Met	Met	Phe	Cys	Leu	Lys	Gln	Lys	Asn	Ser
			340					345					350		
Lys	Lys	Ile	Asn	Ser	His	Arg	Trp	Leu	Phe	Asn	Ala	Phe	Gly	Arg	Ile
		355					360					365			
Leu	Asn	Pro	Glu	Val	Cys	Ile	Leu	Leu	Asp	Ala	Gly	Thr	Lys	Pro	Gly
	370					375					380				
Pro	Lys	Ser	Leu	Leu	Tyr	Leu	Trp	Glu	Ala	Phe	Tyr	Asn	Asp	Lys	Asp
385					390					395					400
Leu	Gly	Gly	Ala	Cys	Gly	Glu	Ile	His	Ala	Met	Leu	Gly	Lys	Gly	Trp
				405					410					415	
Lys	Lys	Leu	Leu	Asn	Pro	Leu	Val	Ala	Ala	Gln	Asn	Phe	Glu	Tyr	Lys
			420					425					430		
Ile	Ser	Asn	Ile	Leu	Asp	Lys	Pro	Leu	Glu	Ser	Ser	Phe	Gly	Tyr	Val
		435					440					445			
Ser	Val	Leu	Pro	Gly	Ala	Phe	Ser	Ala	Tyr	Arg	Phe	Arg	Ala	Ile	Met
	450					455					460				
Gly	Arg	Pro	Leu	Glu	Gln	Tyr	Phe	His	Gly	Asp	His	Thr	Leu	Ser	Lys
465					470					475					480
Gln	Leu	Gly	Lys	Lys	Gly	Ile	Glu	Gly	Met	Asn	Ile	Phe	Lys	Lys	Asn
				485					490					495	
Met	Phe	Leu	Ala	Glu	Asp	Arg	Ile	Leu	Cys	Phe	Glu	Leu	Val	Ala	Lys
			500					505					510		
Ala	Gly	Ser	Lys	Trp	His	Leu	Ser	Tyr	Val	Lys	Ala	Ser	Lys	Gly	Glu
		515					520						525		
Thr	Asp	Val	Pro	Glu	Gly	Ala	Pro	Glu	Phe	Ile	Ser	Gln	Arg	Arg	Arg
	530					535					540				
Trp	Leu	Asn	Gly	Ser	Phe	Ala	Ala	Gly	Ile	Tyr	Ser	Leu	Met	His	Phe
545					550					555					560
Gly	Arg	Met	Tyr	Lys	Ser	Gly	His	Asn	Ile	Val	Arg	Met	Phe	Phe	Leu
				565					570					575	
His	Leu	Gln	Met	Leu	Tyr	Asn	Trp	Phe	Ser	Thr	Phe	Leu	Thr	Trp	Phe
			580					585					590		
Ser	Leu	Ala	Ser	Tyr	Trp	Leu	Thr	Thr	Ser	Val	Ile	Met	Asp	Leu	Val
		595					600					605			
Gly	Thr	Pro	Ser	Ser	Ser	Asn	Gly	Tyr	Thr	Ala	Phe	Pro	Phe	Gly	Lys
	610					615					620				
Thr	Ala	Thr	Pro	Ile	Ile	Asn	Thr	Leu	Val	Lys	Tyr	Ile	Tyr	Leu	Ala
625					630					635					640
Phe	Leu	Leu	Leu	Gln	Phe	Ile	Leu	Ala	Leu	Gly	Asn	Arg	Pro	Lys	Gly
				645					650					655	

Ser Lys Leu Ser Tyr Leu Ala Ser Phe Val Ala Phe Gly Ile Ile Gln
 660 665 670
 Leu Tyr Val Val Val Asp Ala Leu Tyr Leu Val Val Arg Ala Phe Thr
 675 680 685
 Gly Gly Ala Pro Met Asp Phe Asn Thr Asp Asp Gly Ile Gly Ala Phe
 690 695 700
 Leu Ser Ser Phe Phe Gly Ser Ser Gly Ala Gly Ile Ile Ile Ala
 705 710 715 720
 Leu Ala Ala Thr Phe Gly Leu Tyr Phe Val Ala Ser Phe Met Tyr Leu
 725 730 735
 Asp Pro Trp His Met Phe Thr Ser Phe Pro Ala Tyr Met Ala Val Gln
 740 745 750
 Ser Ser Tyr Ile Asn Ile Leu Asn Val Tyr Ala Phe Ser Asn Trp His
 755 760 765
 Asp Val Ser Trp Gly Thr Lys Gly Ser Asp Lys Ala Asp Ala Leu Pro
 770 775 780
 Ser Ala Lys Thr Thr Gly Lys Gly Glu Glu Ala Val Ile Glu Glu
 785 790 795 800
 Ile Asp Lys Pro Gln Ala Asp Ile Asp Ser Gln Phe Glu Ala Thr Val
 805 810 815
 Lys Arg Ala Leu Thr Pro Tyr Val Pro Pro Glu Glu Lys Glu Glu Lys
 820 825 830
 Ser Leu Asp Asp Ser Tyr Lys Ser Phe Arg Thr Arg Leu Val Thr Leu
 835 840 845
 Trp Leu Phe Ser Asn Gly Leu Leu Ala Val Cys Ile Thr Ser Glu Gly
 850 855 860
 Leu Asp Lys Phe Gly Phe Thr Asn Thr Ser Thr Glu Arg Thr Ser Arg
 865 870 875 880
 Phe Phe Gln Ala Leu Trp Ser Asn Ala Val Val Ala Leu Ile Arg
 885 890 895
 Phe Ile Gly Ala Thr Trp Phe Leu Gly Lys Thr Gly Leu Leu Cys Cys
 900 905 910
 Phe Ala Arg Arg
 915

(2) INFORMATION FOR SEQ ID NO:59:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 911 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:59:

Met Ala Tyr Gln Gly Ser Gly Ser His Ser Pro Pro His Tyr Asp Asp
 1 5 10 15
 Asn Gly His Arg Leu Gln Asp Leu Pro His Gly Ser Tyr Glu Glu Glu
 20 25 30
 Ala Ser Arg Gly Leu Leu Ser His Gln Gln Gly Pro Phe Thr Gly Pro
 35 40 45
 Phe Asp Asp Pro Gln Gln His Gly Ser Ser Thr Thr Arg Pro Val Ser
 50 55 60
 Gly Tyr Ser Leu Ser Glu Thr Tyr Ala Pro Glu Ala Ala Tyr His Asp
 65 70 75 80
 Pro Tyr Thr Gln Pro Ser Pro Gly Ser Val Tyr Ser Ala Gln Ser Ala
 85 90 95
 Glu Asn Pro Ala Ala Ala Phe Gly Val Pro Gly Arg Val Ala Ser Pro
 100 105 110
 Tyr Ala Arg Ser Asp Thr Ser Ser Thr Glu Ala Trp Arg Gln Arg Gln
 115 120 125
 Ala Pro Gly Gly Gly Pro Gly Gly Leu Arg Arg Tyr Ala Thr Arg Lys
 130 135 140

Val Lys Leu Val Gln Gly Ser Val Leu Ser Val Asp Tyr Pro Val Pro
 145 150 155 160
 Ser Ala Ile Gln Asn Ala Ile Gln Ala Lys Tyr Arg Asn Asp Leu Glu
 165 170 175
 Gly Gly Ser Glu Glu Phe Thr His Met Arg Tyr Thr Ala Ala Thr Cys
 180 185 190
 Asp Pro Asn Glu Phe Thr Leu His Asn Gly Tyr Asn Leu Arg Pro Ala
 195 200 205
 Met Tyr Asn Arg His Thr Glu Leu Leu Ile Ala Ile Thr Tyr Tyr Asn
 210 215 220
 Glu Asp Lys Thr Leu Thr Ser Arg Thr Leu His Gly Val Met Gln Asn
 225 230 235 240
 Ile Arg Asp Ile Val Asn Leu Lys Lys Ser Glu Phe Trp Asn Lys Gly
 245 250 255
 Gly Pro Ala Trp Gln Lys Ile Val Val Cys Leu Val Phe Asp Gly Ile
 260 265 270
 Asp Pro Cys Asp Lys Asp Thr Leu Asp Val Leu Ala Thr Ile Gly Val
 275 280 285
 Tyr Gln Asp Gly Val Met Lys Arg Asp Val Asp Gly Lys Glu Thr Val
 290 295 300
 Ala His Ile Phe Glu Tyr Thr Thr Gln Leu Ser Val Thr Pro Asn Gln
 305 310 315 320
 Gln Leu Ile Arg Pro Thr Asp Asp Gly Pro Ser Thr Leu Leu Pro Ser
 325 330 335
 Lys Met Met Phe Cys Leu Lys Gln Lys Asn Ser Lys Lys Ile Asn Ser
 340 345 350
 His Arg Trp Leu Phe Asn Ala Phe Gly Arg Ile Leu Asn Pro Glu Val
 355 360 365
 Cys Ile Leu Leu Asp Ala Gly Thr Lys Pro Gly Pro Lys Ser Leu Leu
 370 375 380
 Ser Leu Trp Glu Ala Phe Tyr Asn Asp Lys Asp Leu Gly Gly Ala Cys
 385 390 395 400
 Gly Glu Ile His Ala Met Leu Gly Lys Gly Trp Lys Asn Leu Ile Asn
 405 410 415
 Pro Leu Val Ala Ala Gln Asn Phe Glu Tyr Lys Ile Ser Asn Ile Leu
 420 425 430
 Asp Lys Pro Leu Glu Ser Ser Phe Gly Tyr Val Ser Val Leu Pro Gly
 435 440 445
 Ala Phe Ser Ala Tyr Arg Phe Arg Ala Ile Met Gly Arg Pro Leu Glu
 450 455 460
 Gln Tyr Phe His Gly Asp His Thr Leu Ser Lys Gln Leu Gly Lys Lys
 465 470 475 480
 Gly Ile Glu Gly Met Asn Ile Phe Lys Lys Asn Met Phe Leu Ala Glu
 485 490 495
 Asp Arg Ile Leu Cys Phe Glu Leu Val Ala Lys Ala Gly Ser Lys Trp
 500 505 510
 His Leu Thr Tyr Val Lys Ala Ser Lys Ala Glu Thr Asp Val Pro Glu
 515 520 525
 Gly Ala Pro Glu Phe Ile Ser Gln Arg Arg Arg Trp Leu Asn Gly Ser
 530 535 540
 Phe Ala Ala Gly Ile Tyr Ser Leu Met His Phe Gly Arg Met Tyr Lys
 545 550 555 560
 Ser Gly His Asn Ile Val Arg Met Phe Phe Leu His Ile Gln Met Leu
 565 570 575
 Tyr Asn Ile Phe Ser Thr Val Leu Thr Trp Phe Ser Leu Ala Ser Tyr
 580 585 590
 Trp Leu Thr Thr Thr Val Ile Met Asp Leu Val Gly Thr Pro Ser Asp
 595 600 605
 Asn Asn Gly Asn Lys Ala Phe Pro Phe Gly Lys Thr Ala Thr Pro Ile
 610 615 620
 Ile Asn Thr Ile Val Lys Tyr Val Tyr Leu Gly Phe Leu Leu Leu Gln
 625 630 635 640
 Phe Ile Leu Ala Leu Gly Asn Arg Pro Lys Gly Ser Lys Phe Ser Tyr
 645 650 655
 Leu Ala Ser Phe Val Val Phe Gly Ile Ile Gln Val Tyr Val Val Ile
 660 665 670

Asp	Ala	Leu	Tyr	Leu	Val	Val	Arg	Ala	Phe	Ser	Gly	Ser	Ala	Pro	Met
		675					680					685			
Asp	Phe	Thr	Thr	Asp	Gln	Gly	Val	Gly	Glu	Phe	Leu	Lys	Ser	Phe	Phe
	690					695					700				
Ser	Ser	Ser	Gly	Ala	Gly	Ile	Ile	Ile	Ile	Ala	Leu	Ala	Ala	Thr	Phe
	705				710					715					720
Gly	Leu	Tyr	Phe	Val	Ala	Ser	Phe	Met	Tyr	Leu	Asp	Pro	Trp	His	Met
				725					730					735	
Phe	Thr	Ser	Phe	Pro	Ala	Tyr	Met	Cys	Val	Gln	Ser	Ser	Tyr	Ile	Asn
				740				745					750		
Ile	Leu	Asn	Val	Tyr	Ala	Phe	Ser	Asn	Trp	His	Asp	Val	Ser	Trp	Gly
		755					760					765			
Thr	Lys	Gly	Ser	Asp	Lys	Ala	Asp	Ala	Leu	Pro	Ser	Ala	Lys	Thr	Thr
	770					775					780				
Lys	Asp	Glu	Gly	Lys	Glu	Val	Val	Ile	Glu	Glu	Ile	Asp	Lys	Pro	Gln
	785				790					795					800
Ala	Asp	Ile	Asp	Ser	Gln	Phe	Glu	Ala	Thr	Val	Lys	Arg	Ala	Leu	Thr
				805					810					815	
Pro	Tyr	Val	Pro	Pro	Val	Glu	Lys	Glu	Lys	Thr	Leu	Glu	Asp	Ser	
			820					825				830			
Tyr	Lys	Ser	Phe	Arg	Thr	Arg	Leu	Val	Thr	Phe	Trp	Ile	Phe	Ser	Asn
		835					840					845			
Ala	Phe	Leu	Ala	Val	Cys	Ile	Thr	Ser	Asp	Gly	Val	Asp	Lys	Phe	Gly
	850					855					860				
Phe	Thr	Asn	Ser	Ala	Thr	Asp	Arg	Thr	Gln	Arg	Phe	Phe	Gln	Ala	Leu
	865				870					875					880
Leu	Trp	Ser	Asn	Ala	Val	Val	Ala	Leu	Phe	Arg	Phe	Ile	Gly	Ala	Cys
			885						890				895		
Trp	Phe	Leu	Gly	Lys	Thr	Gly	Leu	Met	Cys	Cys	Phe	Ala	Arg	Arg	
			900					905					910		

(2) INFORMATION FOR SEQ ID NO:60:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:60:

CACCAAGTCA GAGCGTC

17

(2) INFORMATION FOR SEQ ID NO:61:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 17 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:61:

GGCCTTYGAY GAYCCCA

17

(2) INFORMATION FOR SEQ ID NO:62:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:62:

GGGCCGTTTG ACAATCCGCA T

21

(2) INFORMATION FOR SEQ ID NO:63:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 251 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:63:

```

AGGGCCACAG CNTGTGCTAA GCGCCTTGAC GGGGACCCTG GGACTTTCAA GTCTCCTTGG      60
ACCCGGAATT GAATCCTCAC AGAACAGCTT TCAACACTGC TCTAAGGCTG AACTGAGCTG      120
CGCGACTCCG TATCATGGCC AAGACAAATG CTGCTTCAAC TATCCCGGGG GGCAGTTCCT      180
TCAATCGCTG TTTTGGGACG CCGACCCGGC CATTGGACCG GAAGATTCCT GGACTATCCA      240
TGGCTTATGG T                                     251

```

(2) INFORMATION FOR SEQ ID NO:64:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:64:

```

Gly Pro Gln Xaa Val Leu Ser Ala Leu Thr Gly Thr Leu Gly Leu Ser
 1           5           10           15
Ser Leu Leu Gly Pro Gly Ile Glu Ser Ser Gln Asn Ser Phe Gln His
 20           25           30
Cys Ser Lys Ala Glu Leu Ser Cys Ala Thr Pro Tyr His Gly Gln Asp
 35           40           45
Lys Cys Cys Phe Asn Tyr Pro Gly Gly Gln Phe Leu Gln Ser Leu Phe
 50           55           60
Trp Asp Ala Asp Pro Ala Ile Gly Pro Glu Asp Ser Trp Thr Ile His
 65           70           75           80
Gly Leu Trp

```

(2) INFORMATION FOR SEQ ID NO:65:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 83 amino acids
- (B) TYPE: amino acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: None

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:65:

```

Gly Pro Gln Xaa Val Leu Ser Ala Leu Thr Gly Thr Leu Gly Leu Ser
 1           5           10           15
Ser Leu Leu Gly Pro Gly Ile Glu Ser Ser Gln Asn Ser Phe Gln His
 20           25           30
Cys Ser Lys Ala Glu Leu Ser Cys Ala Thr Pro Tyr His Gly Gln Asp
 35           40           45
Lys Cys Cys Phe Asn Tyr Pro Gly Gly Gln Phe Leu Gln Ser Leu Phe
 50           55           60

```

Trp Asp Ala Asp Pro Ala Ile Gly Pro Glu Asp Ser Trp Thr Ile His
 65 70 75 80
 Gly Leu Trp

(2) INFORMATION FOR SEQ ID NO:66:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 214 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:66:

CTACTGAACG	CTTAAAGGTG	CTTAAGGAGC	AACTTCATAT	TATGCGCGAC	CAACGGATCC	60
AGGAAGTCTT	GAGCAATAAG	AAGGGTCGAA	CGCAGCACGG	ACACTCGCAC	AAGCCGACCG	120
GTTTTGGGGG	ACTCAACGGT	TCTCGGCTAA	AGGAGGCCTT	TGTGGGACGT	CGAATCGGGA	180
AGAATTCCAA	GGCATTGGCC	GAATTGGCCA	CCCC			214

(2) INFORMATION FOR SEQ ID NO:67:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:67:

GTTCTATTGA	GATACGCG	18
------------	----------	----

(2) INFORMATION FOR SEQ ID NO:68:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:68:

ACAAGCCGAC	CGGTTTTG	18
------------	----------	----

(2) INFORMATION FOR SEQ ID NO:69:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:69:

CGATAAGGAC	TCCAAGAG	18
------------	----------	----

(2) INFORMATION FOR SEQ ID NO:70:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:70:

GTCGCGCATA ATATGAAG

18

(2) INFORMATION FOR SEQ ID NO:71:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 336 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:71:

AGCACCTATA ATCTATGCTG TCCCACTATC ACACATCTAT ATGTTGTACA AGCCTGATAC	60
AATCAATAAT GATGTAATAA TTGACTCTGG AAAGTTGGCT ATAAAACTCA CCATACAAGT	120
CCAGATAACC CTGCCAAACT CCACTCCCAG GGCATTAATC TTCATTTATA TCGACCAGCC	180
ATACCTATGG TCAAATCACA CGCAACGCCA CAGATATATA TTTGAATCAA ATTTCTCTTT	240
TGAAGAAGAA AGGGTGGTTT ATGAGGAAGA ATATCCCAAT ATGCCAATCT GACTGTTCCG	300
GATTGGAATA ATGCACAAGC TTCGAATATA AATATA	336

(2) INFORMATION FOR SEQ ID NO:72:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:72:

CTTCCTCATA AACCACCC

18

(2) INFORMATION FOR SEQ ID NO:73:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:73:

AACTGACAGG ACAAGACC

18

(2) INFORMATION FOR SEQ ID NO:74:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:74:

GACTTGCATC ACTTCCTC

18

(2) INFORMATION FOR SEQ ID NO:75:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 18 base pairs
- (B) TYPE: nucleic acid

- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:75:

TGAAGCTGAG AGTAGGTG

18

(2) INFORMATION FOR SEQ ID NO:76:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 281 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:76:

CAAGGGAACG	GGAATAAAAT	ACACATAACA	AAGGATTCGA	AGAAAGAAAA	AAAAAGGGGG	60
GGAGGTGTGT	CCAAGAGGAA	AGAAGAAAAA	AAATTTAATT	TCGCCACCCT	ATCGCGGAGT	120
GTTCCGCCTT	CAGGAGAGAT	AGAAAAGAGG	AGGAGAAGG	GAGAAGGAAA	AAAAAAAACA	180
GAATTTCCAC	AGACAAGGAA	AGCTTAACCG	GGTCACGAAA	AAGCACAATA	CAGGTGAACA	240
ACTGAGGGGA	AGGGGGCCAA	AAAGAAAAAA	ATAATTCCTA	A		281

(2) INFORMATION FOR SEQ ID NO:77:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:77:

GTTTCGGTAT TGTCACTG

18

(2) INFORMATION FOR SEQ ID NO:78:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:78:

ACAGGTGAAC AACTGAGG

18

(2) INFORMATION FOR SEQ ID NO:79:

- (i) SEQUENCE CHARACTERISTICS:
 - (A) LENGTH: 18 base pairs
 - (B) TYPE: nucleic acid
 - (C) STRANDEDNESS: single
 - (D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:79:

CGACCAAAC AGACAAGC

18

(2) INFORMATION FOR SEQ ID NO:80:

- (i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 18 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:80:

CTTTCCTCTT GGACACAC

18

CLAIMS

What is claimed is:

5 1. A method of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

10 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates transcription, translation or secretion of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the
15 conditions; and

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

20

2. The method of claim 1, wherein the nucleic acid construct has less than 40% homology with the first DNA sequence.

3. The method of claim 1, wherein the nucleic acid construct has less than 40%
25 homology with the locus.

4. The method of claim 1, wherein the locus is on a different chromosome than the first DNA sequence or on the same chromosome but at least 3,000 bps from the 5' or 3' terminus of the first DNA sequence.

30

5. A method of producing a polypeptide, comprising

(A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

6. A method of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates transcription of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell expresses more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

7. The method of claim 6, wherein the nucleic acid construct has less than 40% homology with the first DNA sequence.

8. The method of claim 6, wherein the nucleic acid construct has less than 40% homology with the locus.

9. The method of claim 6, wherein the locus is on a different chromosome than the first
5 DNA sequence or on the same chromosome but at least 3,000 bps from the 5' or 3' terminus of the first DNA sequence.

10. A method of producing a polypeptide, comprising

10 (A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence,
15 wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell expresses more of the polypeptide than the parent cell
20 when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

11. A method of producing a polypeptide, comprising

25 (a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence,
30 not within a second DNA sequence encoding a protein that negatively regulates

translation of the polypeptide, and not within a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell synthesizes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

5

(b) recovering the polypeptide.

12. The method of claim 11, wherein the nucleic acid construct has less than 40% homology with the first DNA sequence.

10

13. The method of claim 11, wherein the nucleic acid construct has less than 40% homology with the locus.

14. The method of claim 11, wherein the locus is on a different chromosome than the first DNA sequence or on the same chromosome but at least 3,000 bps from the 5' or 3' terminus of the first DNA sequence.

15

15. A method of producing a polypeptide, comprising

20 (A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

25

(ii) the mutant cell synthesizes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

30

(B) recovering the polypeptide.

16. A method of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

5 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence, not within a second DNA sequence encoding a protein that negatively regulates secretion of the polypeptide, and not within a third DNA sequence encoding a
10 protease capable of hydrolyzing the polypeptide under the conditions; and

(ii) the mutant cell secretes more of the polypeptide than the parent cell when both cells are cultivated under the conditions;

(b) recovering the polypeptide.

15

17. The method of claim 16, wherein the nucleic acid construct has less than 40% homology with the first DNA sequence.

18. The method of claim 16, wherein the nucleic acid construct has less than 40%
20 homology with the locus.

19. The method of claim 16, wherein the locus is on a different chromosome than the first DNA sequence or on the same chromosome but at least 3,000 bps from the 5' or 3' terminus of the first DNA sequence.

25

20. A method of producing a polypeptide, comprising

(A) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

30 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence,

wherein the introduction of the nucleic acid construct disrupts a gene encoding an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase, a ligase, or regulatory or control sequences thereof, other than a gene encoding a protease which is capable of hydrolyzing the polypeptide under the conditions; and

- 5 (ii) the mutant cell secretes more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the polypeptide.

10 21. A method of producing a polypeptide, comprising

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

- 15 (i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the random integration of a nucleic acid construct into the genome of the parent cell at a locus wherein the nucleic acid construct is not homologous with the locus and wherein the locus is not within the first DNA sequence nor within a second DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions; and

- 20 (ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

(b) recovering the polypeptide.

25 22. The method of claim 1, wherein the nucleic acid construct is introduced by restriction enzyme-mediated integration.

23. The method of claim 1, wherein the nucleic acid construct comprises a selectable marker.

30 24. The method of claim 23, wherein the selectable marker is *amdS*, *argB*, *bar*, *hygB*, *niaD*, *pyrG*, *sC*, or *trpC*.

25. The method of claim 1, wherein the parent cell is a mammalian cell.
26. The method of claim 1, wherein the parent cell is a bacterial cell.
- 5 27. The method of claim 1, wherein the parent cell is a fungal cell.
28. The method of claim 27, wherein the fungal cell is a filamentous fungal cell.
29. The method of claim 28, wherein the filamentous fungal cell is selected from the
10 group consisting of *Acremonium*, *Aspergillus*, *Fusarium*, *Humicola*, *Mucor*, *Myceliophthora*,
Neurospora, *Penicillium*, *Scytalidium*, *Thielavia*, *Tolypocladium*, and *Trichoderma*.
30. The method of claim 27, wherein the fungal cell is a yeast cell.
- 15 31. The method of claim 1, wherein the polypeptide is a recombinant polypeptide.
32. The method of claim 1, wherein the polypeptide is a heterologous polypeptide.
33. The method of claim 1, wherein the polypeptide is a hormone, a hormone variant,
20 an enzyme, a receptor or portions thereof, an antibody or portions thereof, or a reporter.
34. The method of claim 33, wherein the polypeptide is an oxidoreductase, a transferase,
a hydrolase, a lyase, an isomerase, or a ligase.
- 25 35. The method of claim 34, wherein the polypeptide is an aminopeptidase, an amylase,
a carbohydrase, a carboxypeptidase, a catalase, a cellulase, a chitinase, a cutinase, a
deoxyribonuclease, an esterase, an alpha-galactosidase, a beta-galactosidase, a glucoamylase,
an alpha-glucosidase, a beta-glucosidase, an invertase, a laccase, a lipase, a mannosidase,
a mutanase, an oxidase, a pectinolytic enzyme, a peroxidase, a phytase, a polyphenoloxidase,
30 a proteolytic enzyme, a ribonuclease, or a xylanase.

36. The method of claim 1, wherein the mutant cell has an increased uptake of an inorganic cofactor compared to the parent cell.
37. The method of claim 1, wherein the mutant cell has a more desirable morphology
5 than the parent cell.
38. The method of claim 1, wherein the mutant cell produces higher yields of one or more secreted proteins than the parent cell.
- 10 39. The method of claim 1, wherein the mutant cell which has lost its ability to synthesize one or more essential metabolites.
40. The method of claim 1, wherein a phenotype of the mutant cell is observed only under certain conditions.
15
41. The method of claim 1, wherein the mutant cell exhibits an altered growth rate relative to the parent cell.
42. The method of claim 1, wherein the growth of the mutant cell is not inhibited by the
20 overproduction of a desired polypeptide or metabolite when grown under conditions that induce high level production of the polypeptide or metabolite.
43. The method of claim 1, wherein the mutant cell is able to tolerate lower oxygen conditions than the parent cell.
25
44. The method of claim 1, wherein the mutant cell exhibits altered production of a transcriptional activator of a promoter than the parent cell.
45. The method of claim 1, wherein the mutant cell has a mutation in one or more of the
30 genes of the signal transduction pathway of the parent cell.

46. The method of claim 1, wherein the mutant cell does not recognize and erroneously splice a cryptic intron.

47. The method of claim 1, wherein the nucleic acid construct is pDSY109, pDSY112,
5 pMT1936, pDSY138, pDSY162, pDSY163, pDSY141, pSMO1204, pSMOH603, p4-8.1, p7-14.1, pHB220, pSMO717, pSMO321, pHowB571 or pSMO810.

48. The method of claim 1, wherein the locus is SEQ ID NO:9, SEQ ID NO:16, SEQ ID NO:25, SEQ ID NO:29, SEQ ID NO:34, SEQ ID NO:39, SEQ ID NO:50, SEQ ID
10 NO:56, SEQ ID NO:63, SEQ ID NO:66, SEQ ID NO:71, SEQ ID NO:76, or a fragment thereof.

49. The method of claim 1, wherein the locus encodes a glucose transporter, mannitol-1-phosphate dehydrogenase, chitin synthase, heat shock protein, manganese superoxide
15 dismutase, or a gene required for activation of *pacC*,

50. The method of claim 49, wherein the locus is a *palB* gene.

51. A method of producing a polypeptide, comprising
20

(a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into
25 the genome of the parent cell at a locus which is not within the first DNA sequence and a second DNA sequence encoding a protein that positively regulates transcription, translation or secretion of the polypeptide; and

(ii) the mutant cell produces less of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

30

(b) recovering the polypeptide.

52. A method of producing a metabolite, comprising

(A) cultivating a mutant cell under conditions conducive for production of the metabolite, wherein

- 5 (i) the mutant cell is related to a parent cell, which comprises one or more first DNA sequences encoding first polypeptides in the biosynthetic pathway of the metabolite, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within (a) the first DNA sequences, (b) a second DNA sequence encoding a protein that negatively regulates transcription, translation or secretion of the first polypeptides, (c) a third DNA sequence encoding a protease capable of hydrolyzing any of the first polypeptides under the conditions, and (d) one or more fourth DNA sequences encoding a second polypeptide in the second biosynthetic pathway of a second metabolite wherein the biosynthetic pathway and the second biosynthetic pathway involve the production of the same intermediate and the second polypeptide catalyzes a step after the production of the intermediate; and
- 10 15 (ii) the mutant cell produces more of the metabolite than the parent cell when both cells are cultivated under the conditions; and

(B) recovering the metabolite.

20

53. A method of producing a first polypeptide, comprising

- (a) forming a mutant cell by introducing a nucleic acid construct into the genome of the parent cell, which comprises a first DNA sequence encoding the polypeptide, at a locus which is not within the first DNA sequence, a second DNA sequence encoding a protein that negatively regulates transcription, translation or secretion of a second polypeptide, and a third DNA sequence encoding a protease capable of hydrolyzing the polypeptide under conditions conducive to the production of the first polypeptide;
- 25 (b) isolating the mutant cell which produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions;
- 30 (c) identifying the locus wherein the nucleic acid construct has been integrated;
- (d) producing a cell in which a corresponding locus has been disrupted;
- (e) culturing the cell under the conditions; and

(f) recovering the first polypeptide.

54. A method of producing a polypeptide, comprising

5 (a) cultivating a mutant cell under conditions conducive for production of the polypeptide, wherein

(i) the mutant cell is related to a parent cell, which comprises a first DNA sequence encoding the polypeptide, by the introduction of a nucleic acid construct into the genome of the parent cell at a locus which is not within the first DNA sequence and a second DNA sequence encoding a protease capable of hydrolyzing the polypeptide under the conditions, wherein the introduction of the nucleic acid construct specifically enhances transcription, translation or secretion of the polypeptide; and

10

(ii) the mutant cell produces more of the polypeptide than the parent cell when both cells are cultivated under the conditions; and

15

(b) recovering the polypeptide.

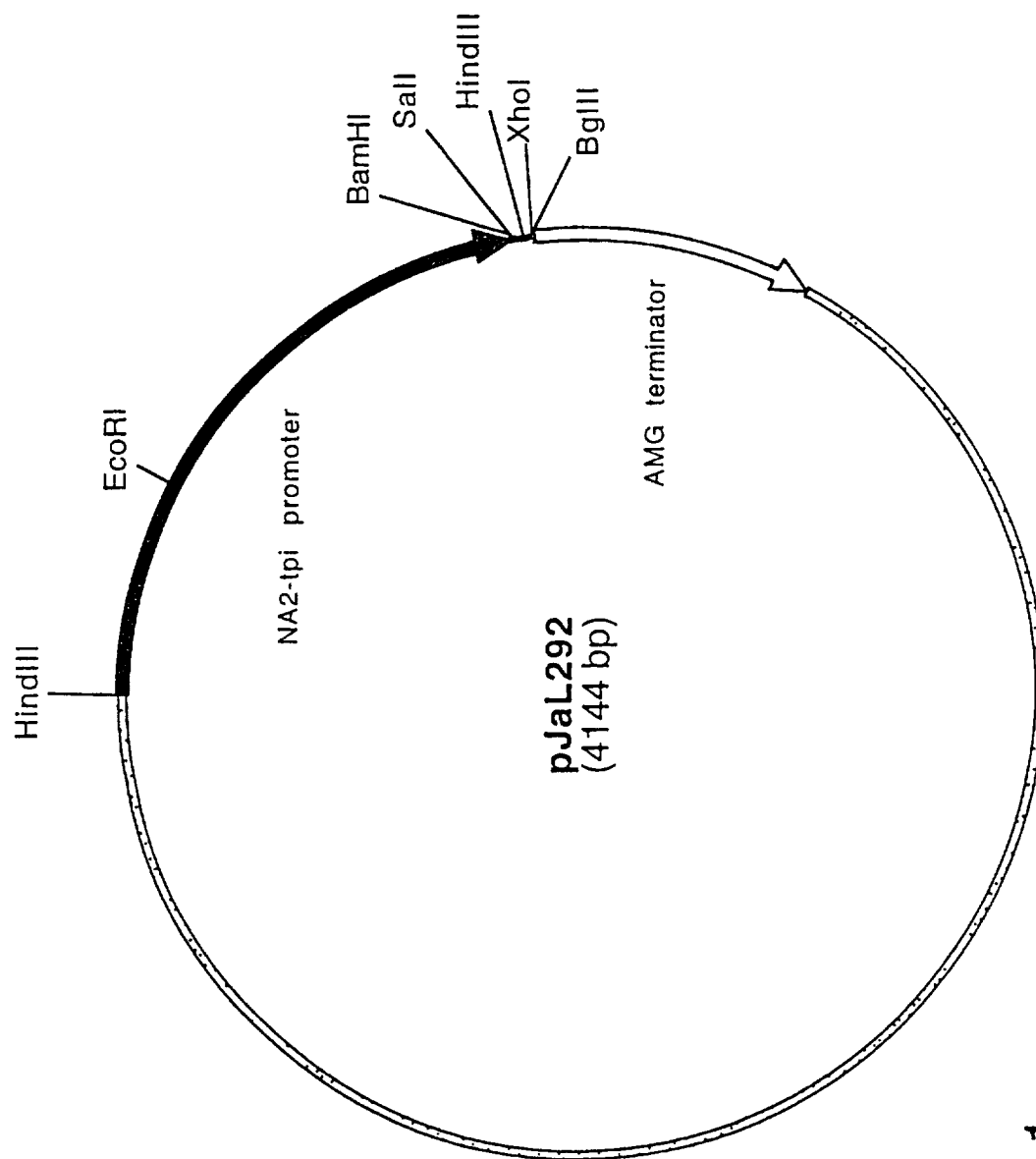


Fig. 1

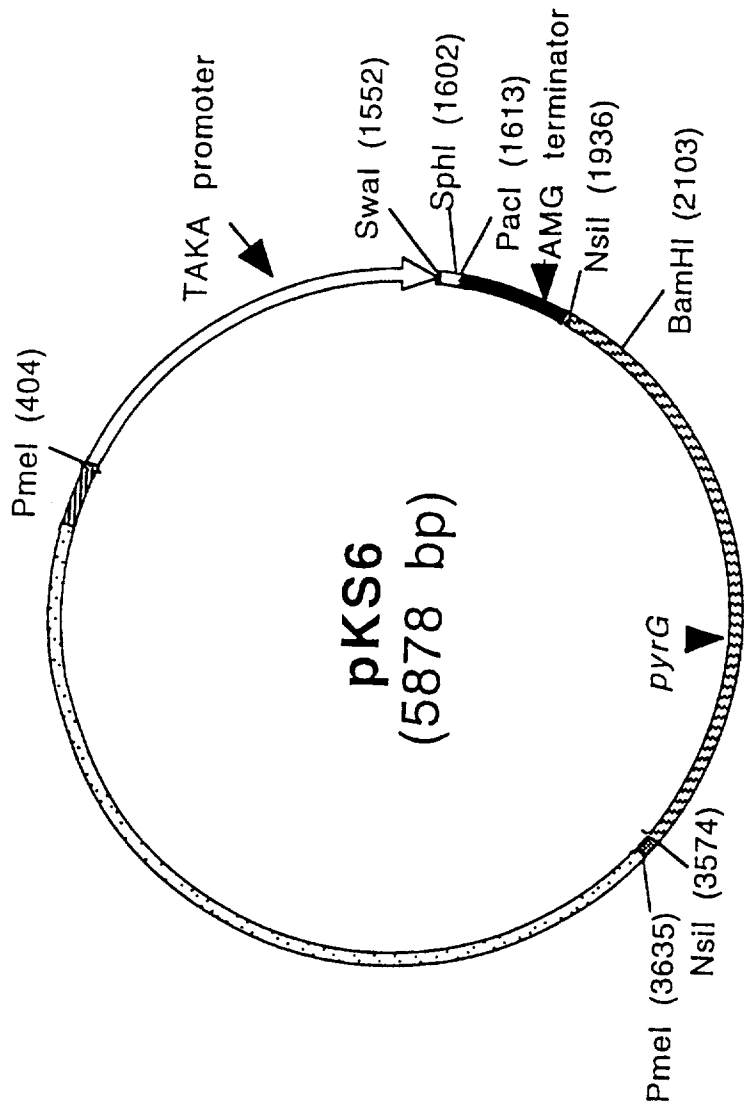


Fig. 2

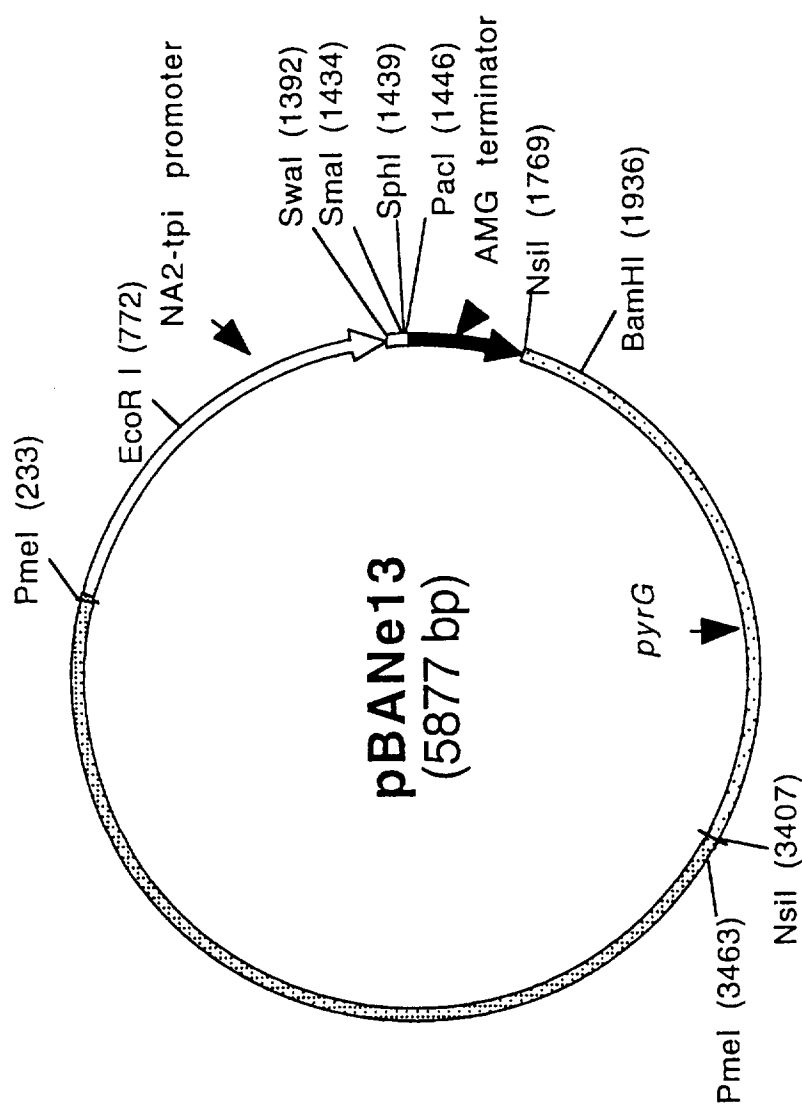


Fig. 3

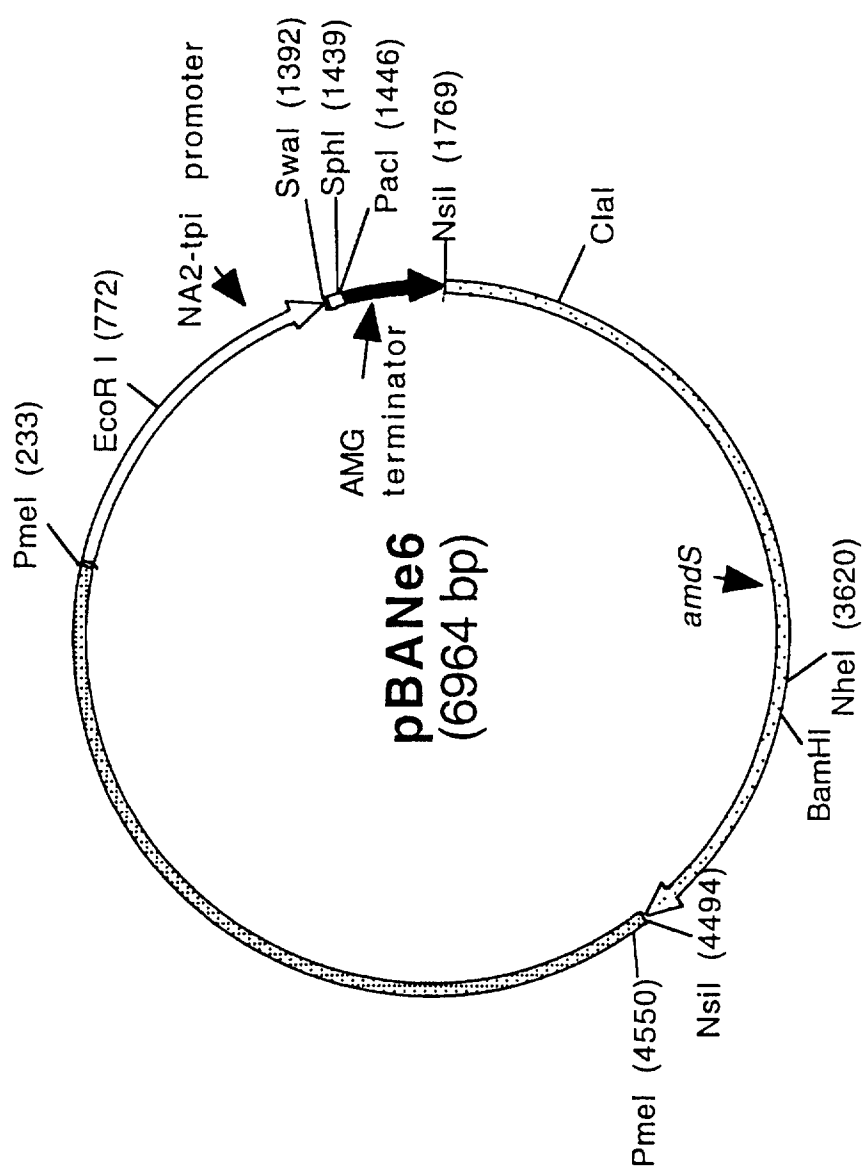


Fig. 4

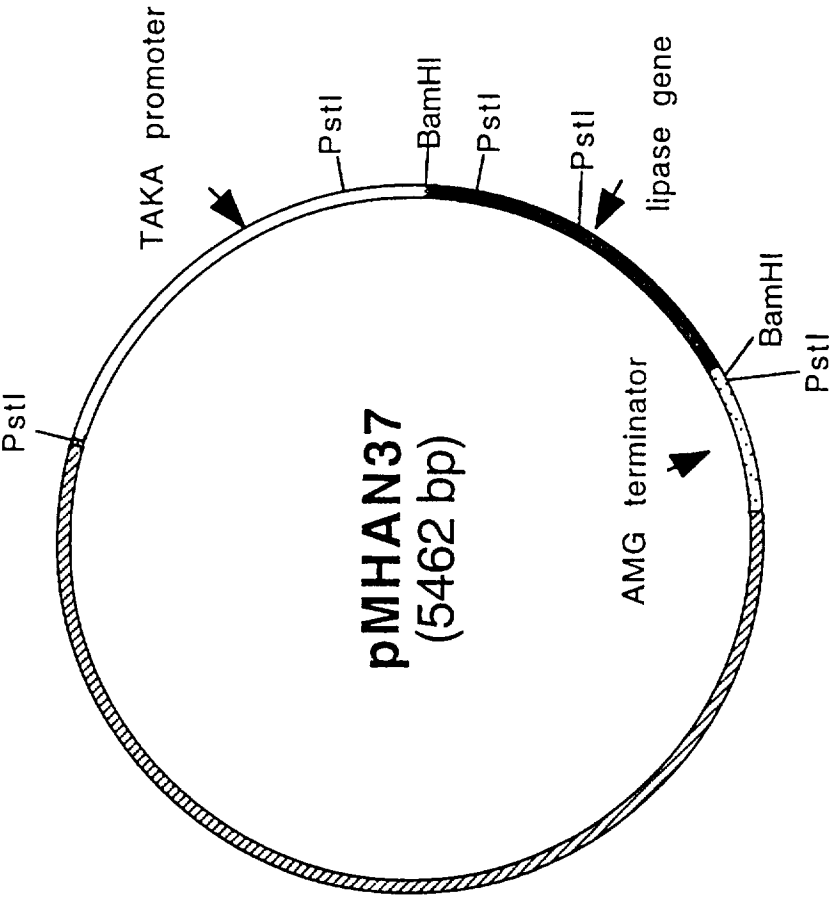


Fig. 5

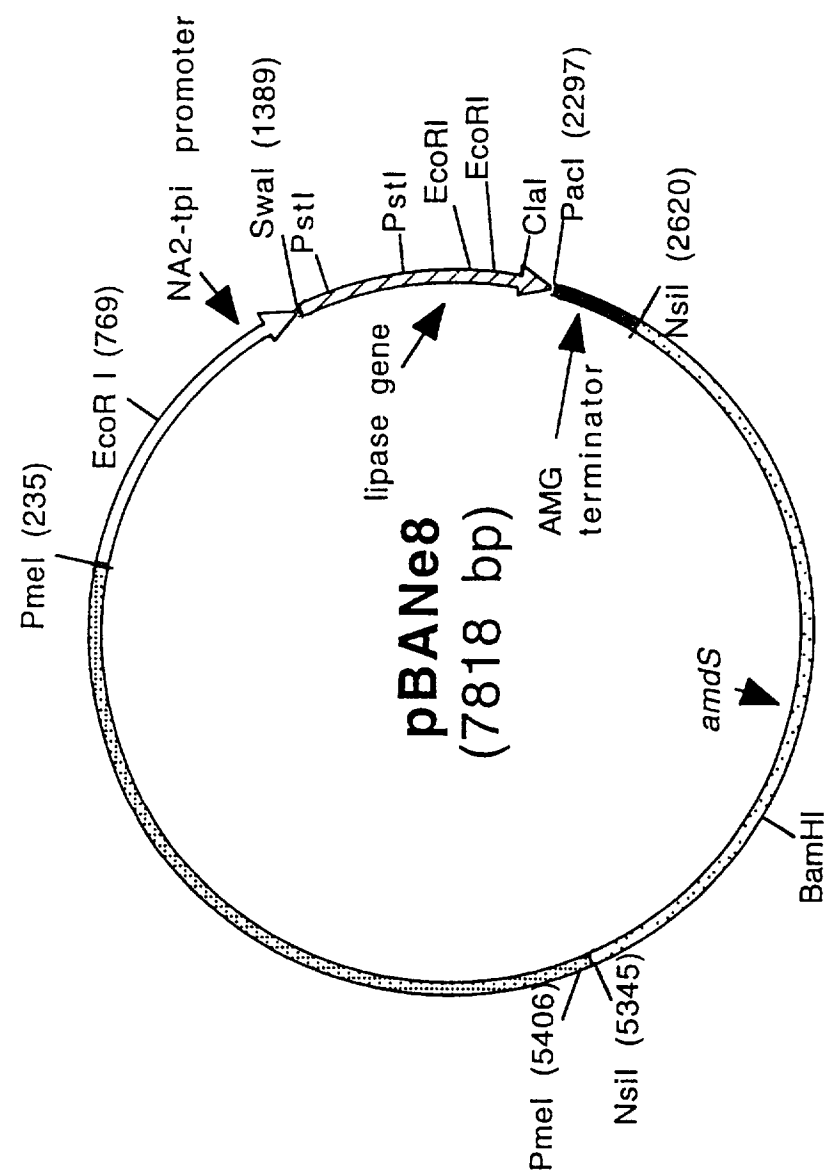


Fig. 6

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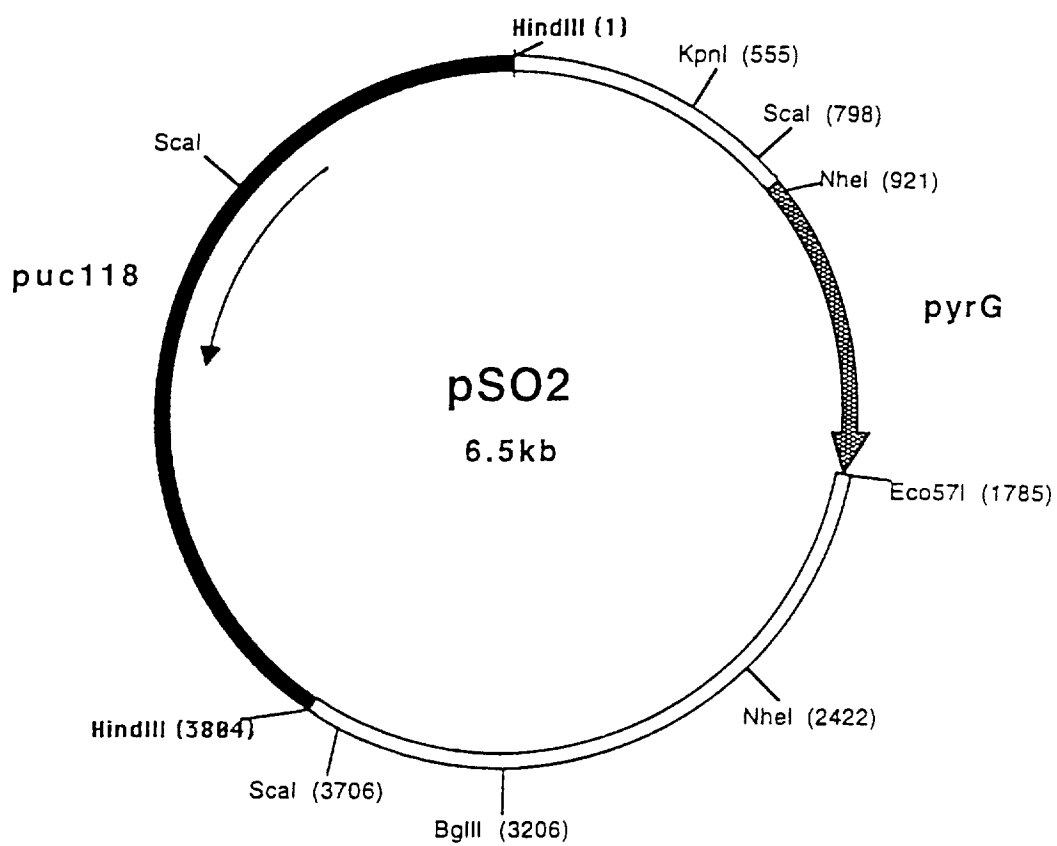


Fig.7

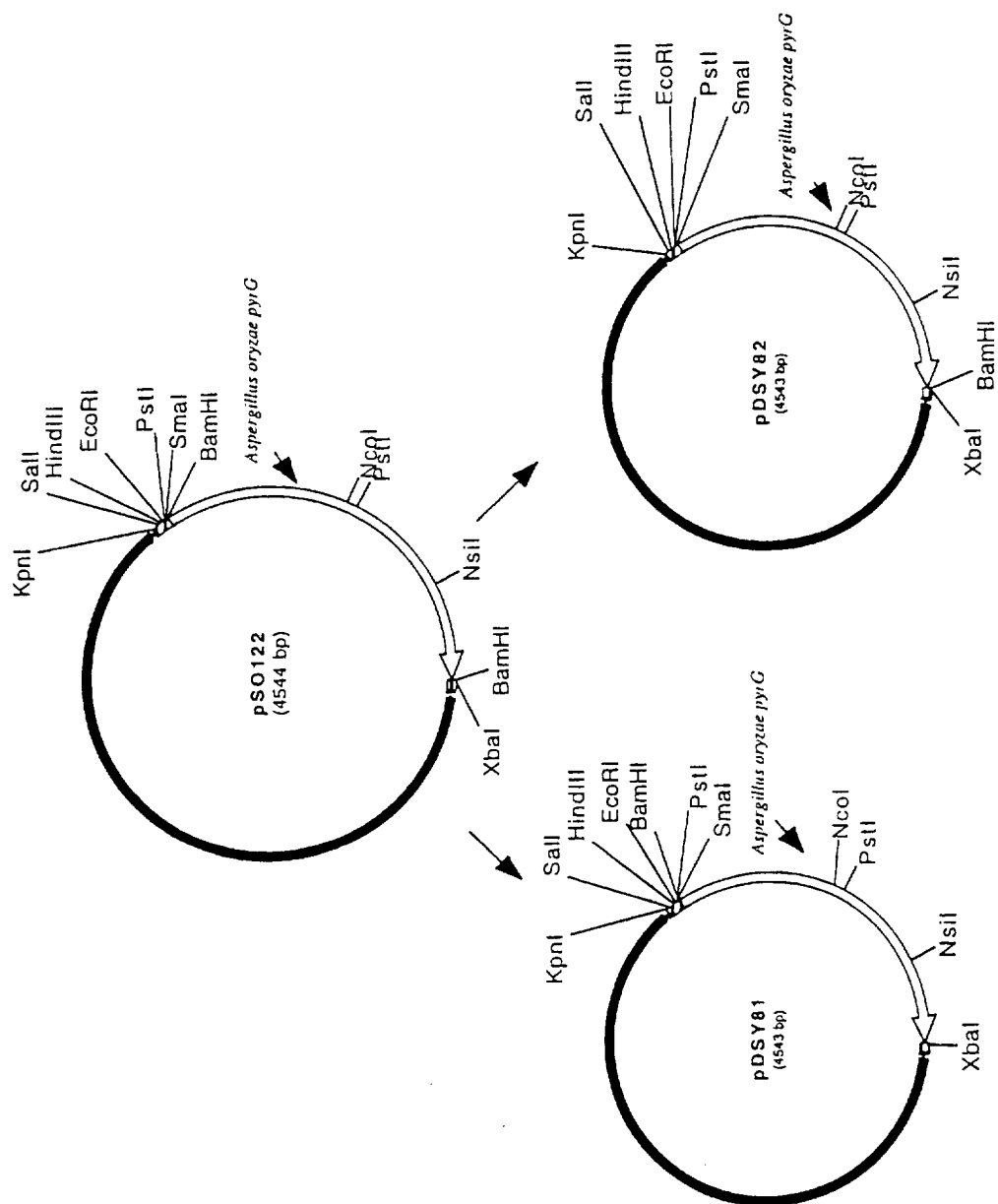


Fig. 8

ACACGGCCCTGGACAATGAGCAACCTCTCTAAAGAGTTGAGATTAGTATATATACTAGCCAGGAGCGGACTTTCCAAATATTATGTAATTAAGTGTGTTGTAAGTGTACCTATAA 120
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 CTACGATAAATATGCAAGGGTCACTTAAACAACTATACCTGGACAAAAGTATGGAGAAACAATAGATTAAAGAGTCCGTTCTATATTTAATCCCTCCGCGGAGGTTTCCTT 360
 ATCCGATAGATACGATAGGATCCAGTAAGCTCATTCATCCCGTGAGATAAGAGGACTCCACCTCAACAACTAATCATCAAACTCCAAATCAATATCAATGAACCCATAACAACIT 480
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 G P L Q F G Y H L A E L N A P O A V I
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 T C E R K S I H S T T R G L P Q C I P M N P S O F G L V S S I Y T L G L L G
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 TAATTCGCTCTAGCTAGCTATCTTCAACCTGAAGCTTATCCGCTAGCAGTCTTCTTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 1800
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 CCAGCGCAGACAGGACTAATTTTCTGGCTCTTTGATTTTTTCTGGGCTTACTCTGCTGCCAACATTCAGATTATCAATAGTAGTCAATCTGTGACTATCTCTCCGAGGGAT 2160
 Q R R Q D
 AGCTGCAAGGTGTGACCTCCACAGAGGAATCTATCTGTGAGATCAAGAGCAATAGATAGCAATTAATTTGGTGTCTCTACCTAGGAGCATTCGGTGAGAGTCAAGAGTCATAC 2280
 TTGCTCTGGCTGTGTCATCCCGAGTCACTAGTCAGGTTAGTCTGGCAGTAAAGAGCAATCCCGTCTACTTCCATCTCAAACTGTACCGGAAACAAGAGTAAGAGGGGTGATGA 2400
 TACCTCTAAATATGATAGTGTGACATGCTCTTATACCAACCCCTGGAAGAGTCTTGTATGATCTACACAGGAGTGTGATCATCTGCGATCGAGTCACTTCGCGAC 2520
 CGCACACTGCATGACAGTGGGGGCGAGGGGCGCAAGGCGCAAAATTTAGTGATTTGAAGCAATTTGTATACACCTTTTACCCCTCAATCCATGATAGGGAAGAGAGATGCT 2640
 GAGAGAGGCTACTGCCACGCTAGACTGGATTGGTCCGTATATGACGCTATGACGCGACAGGGGGCTGCTTCTCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCTGCT 2760
 TAGATAGATTGGCGTGGGTCAGGATATGATAGAGACAAATGAGGTTGGGCTTTCGGAATAGGGAAGAGTGTGGACAGATGAAGAAACGCTGCTGCTGCTGCTGCTGCTGCTGCT 2880
 TTGAGATATTTTAAGTGTGCTGATGATTTTGGCAAGGCTGAGACATTTATCTAGGTAATTTCCAGAAAGCACTTAGGGAAGAGTAAAGCCACAACGAGATACGTGAGAGGAG 3000

Fig. 9

[illegible]

Fig. 10

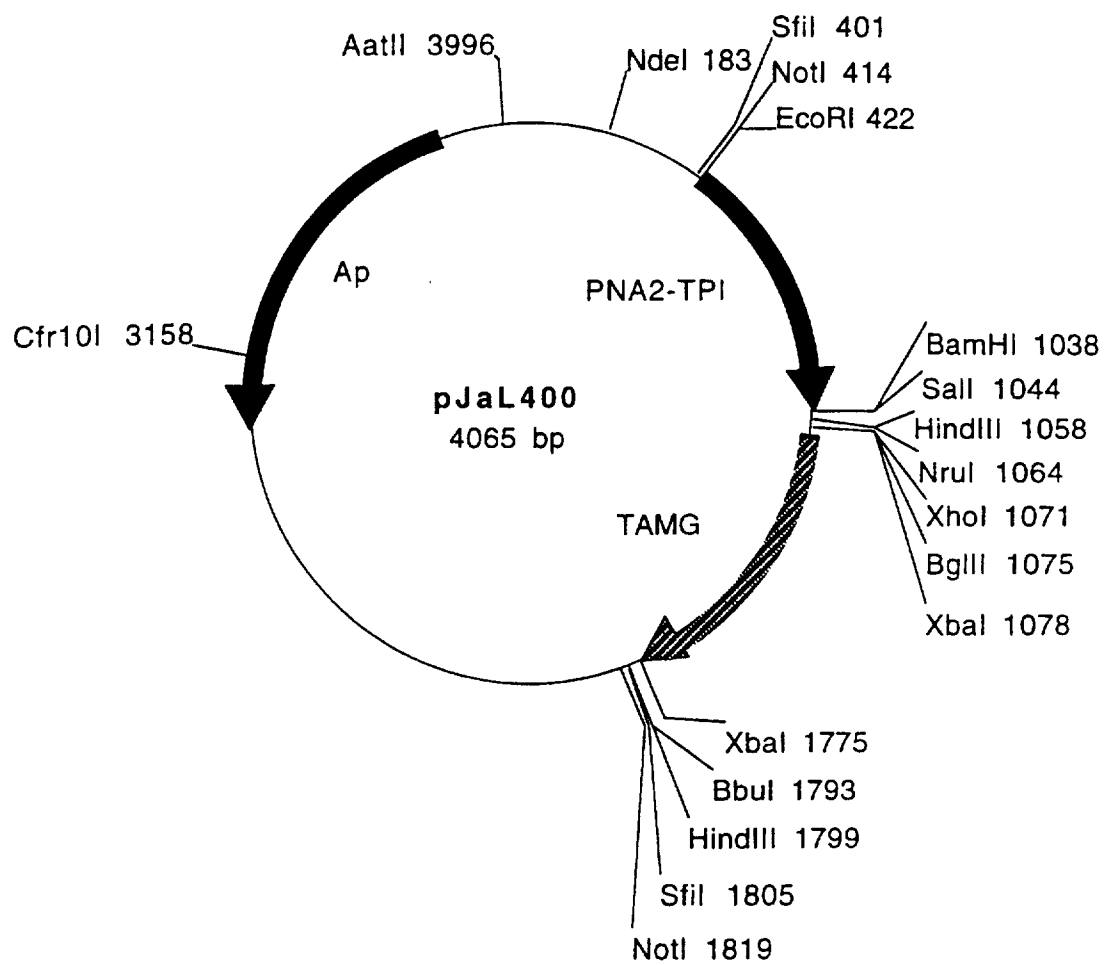


Fig. 11

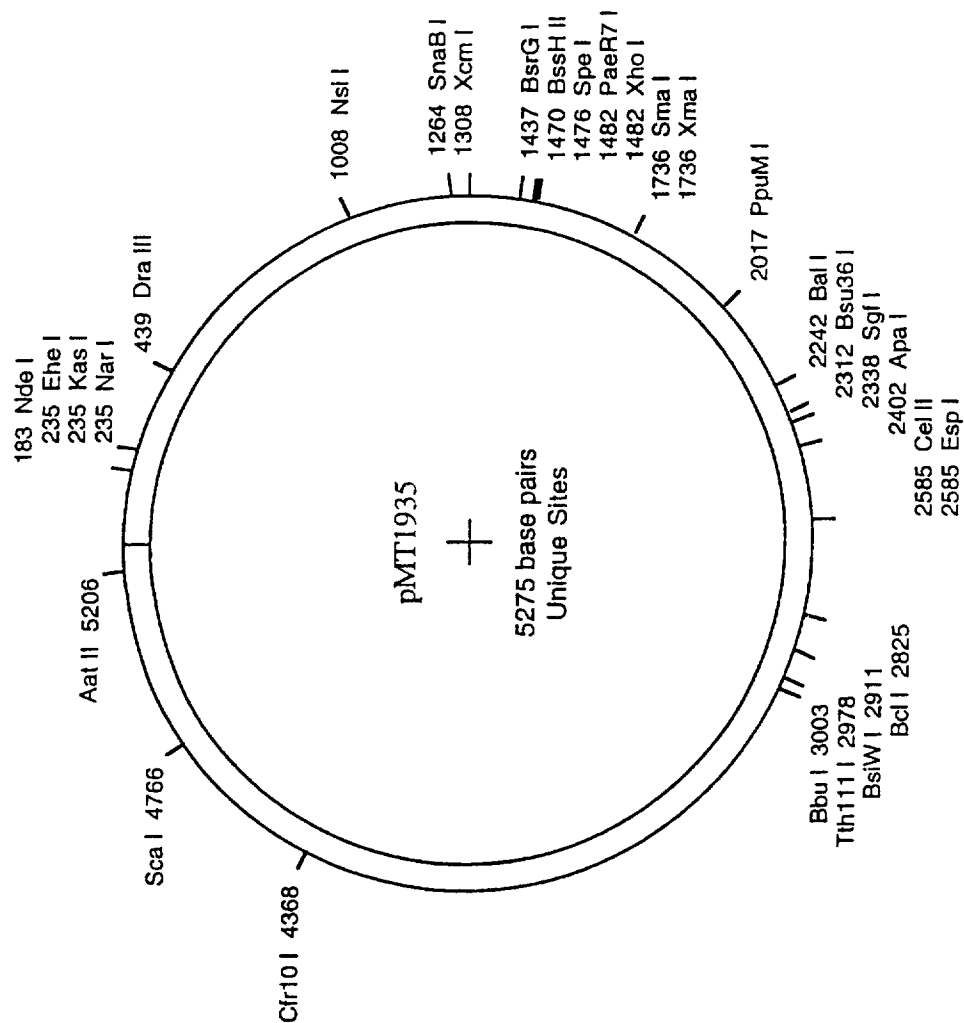


Fig. 12

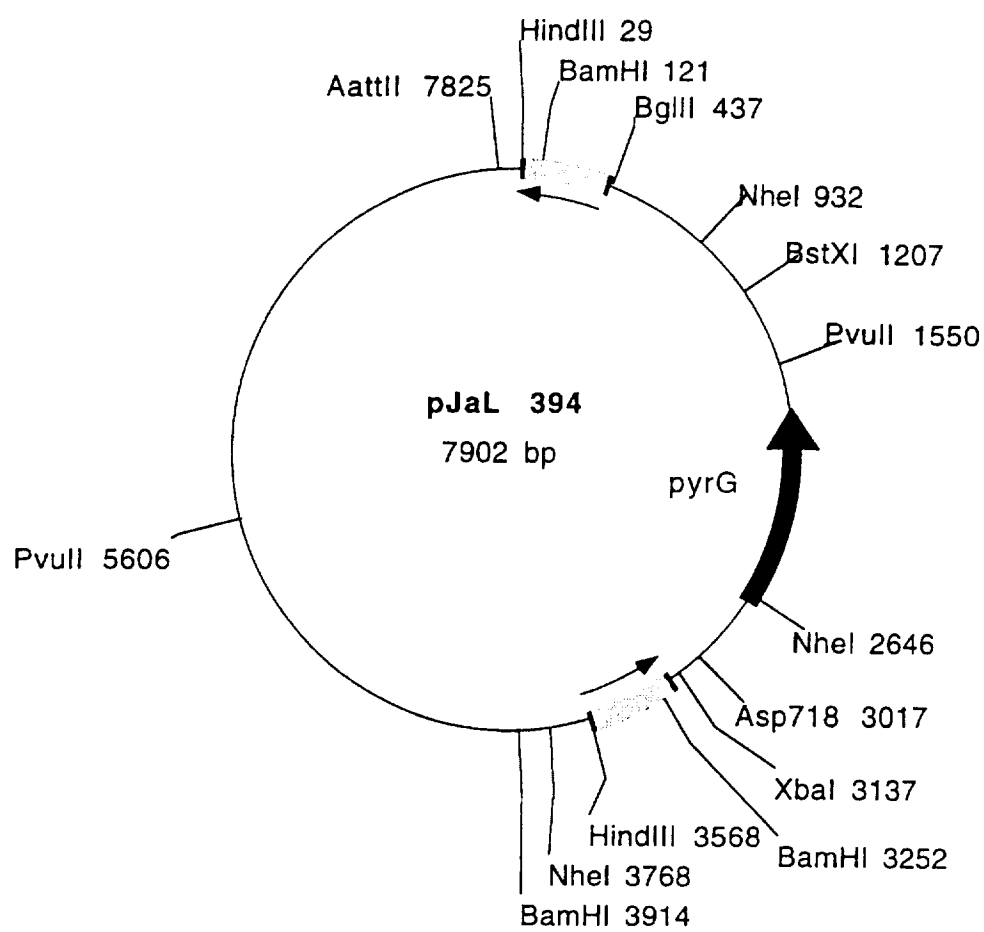


Fig. 13

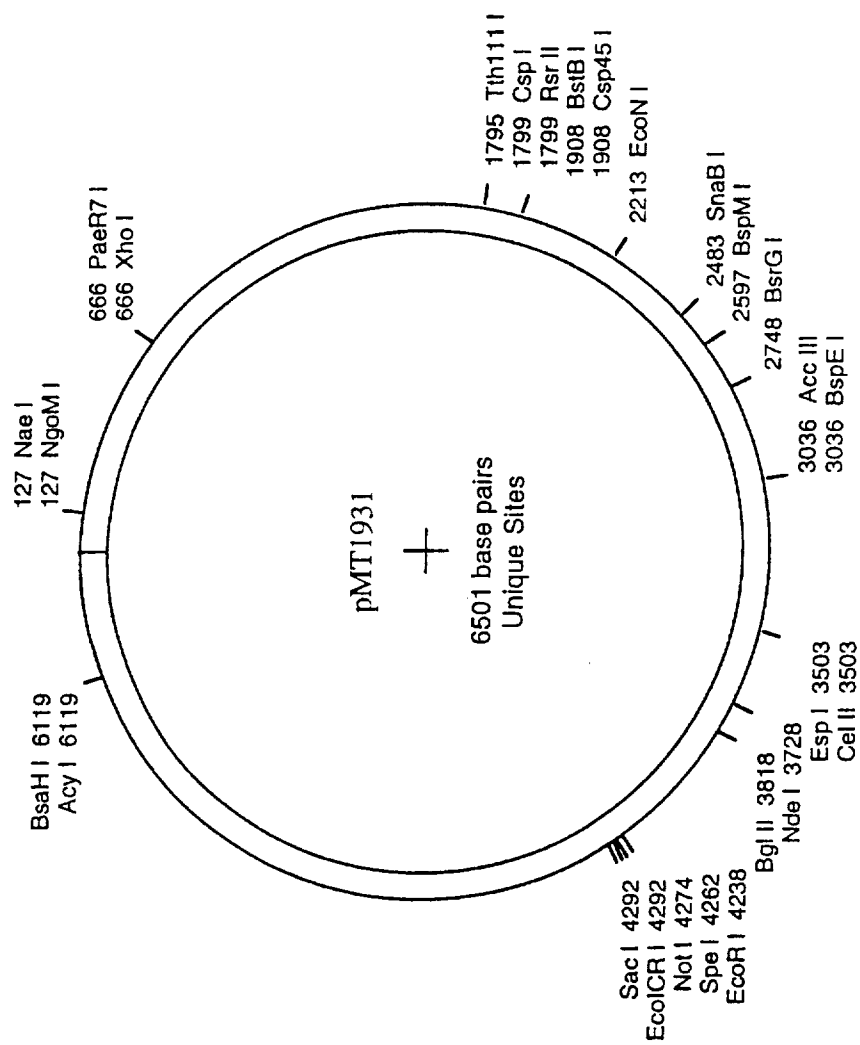


Fig. 14

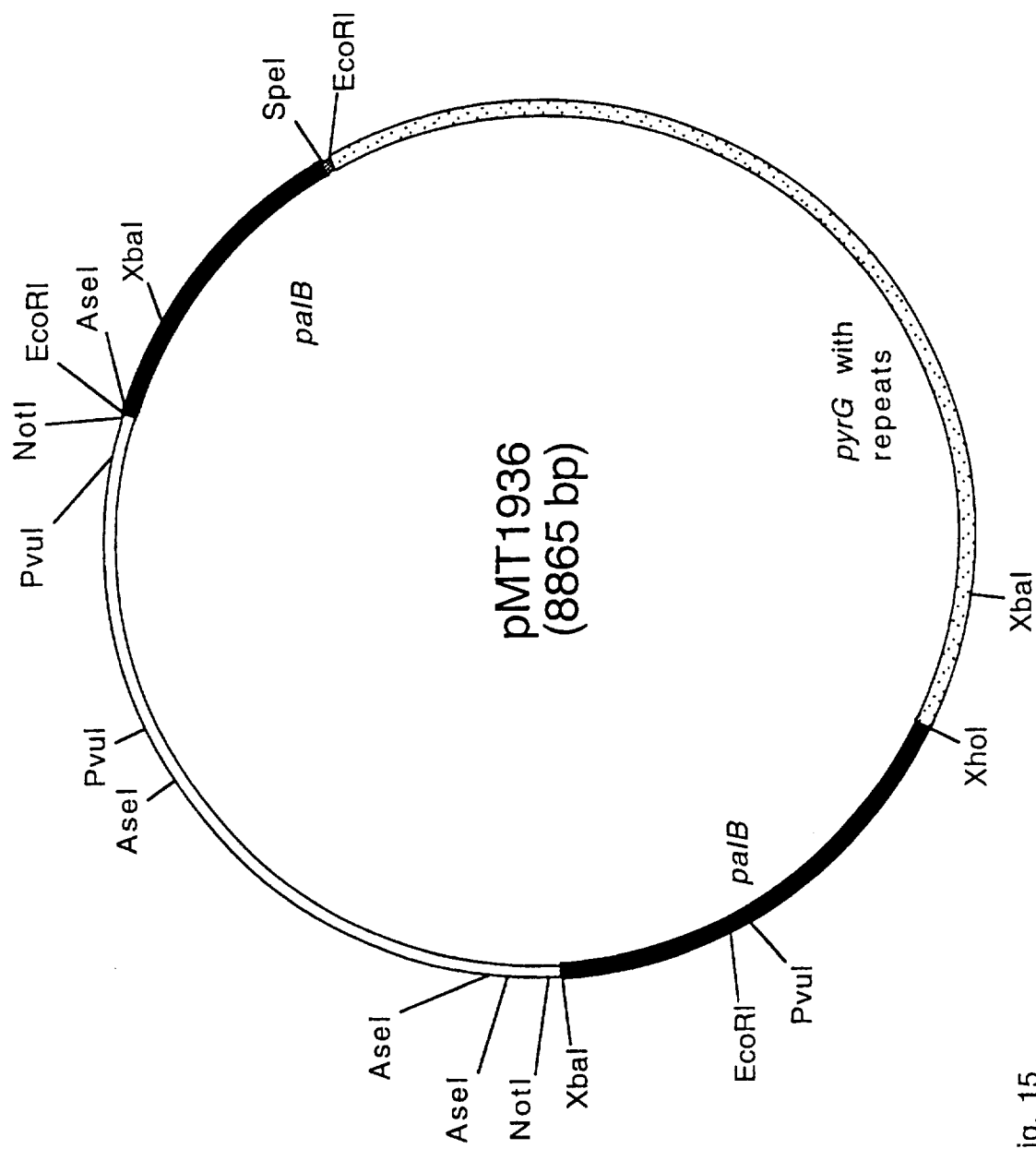


Fig. 15

[illegible]

Fig. 16

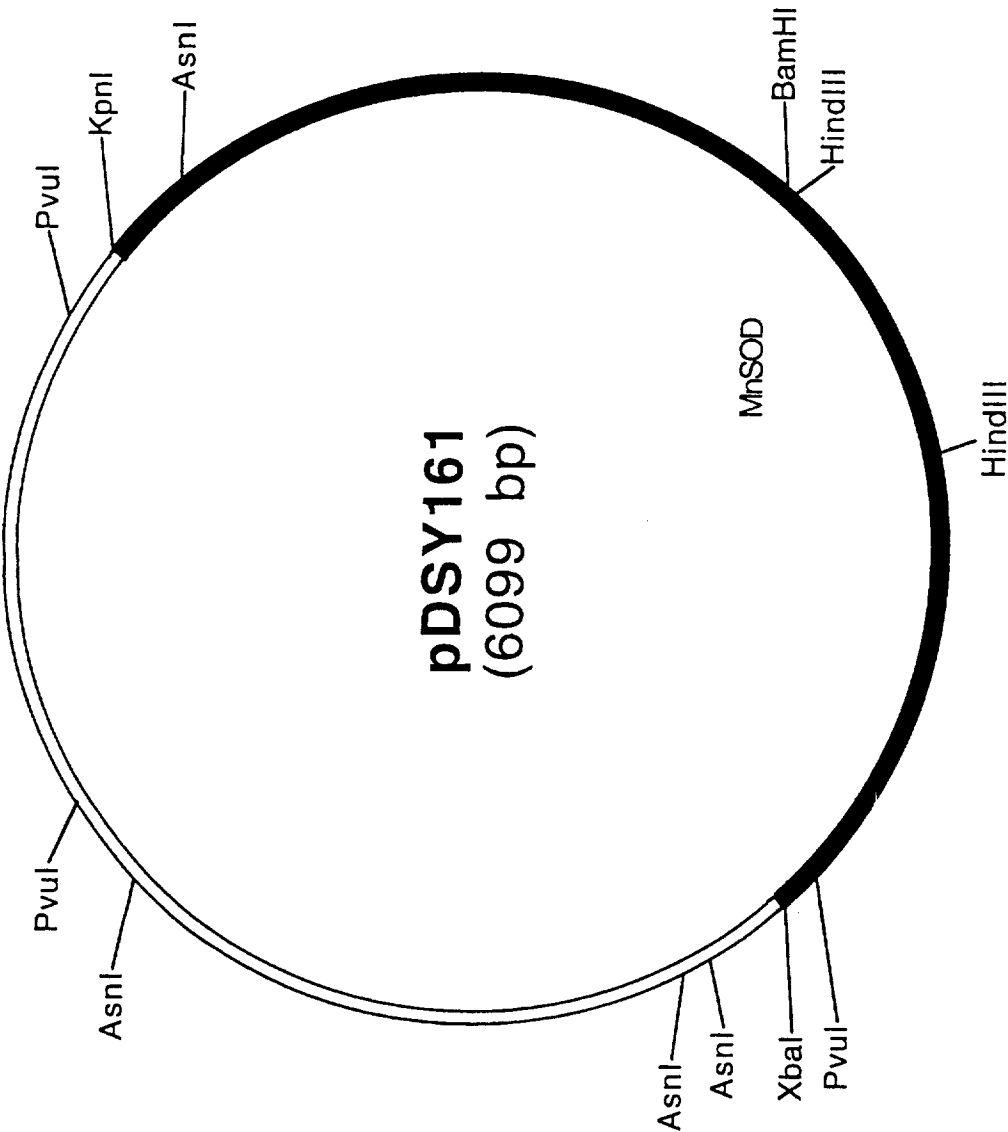


Fig. 18

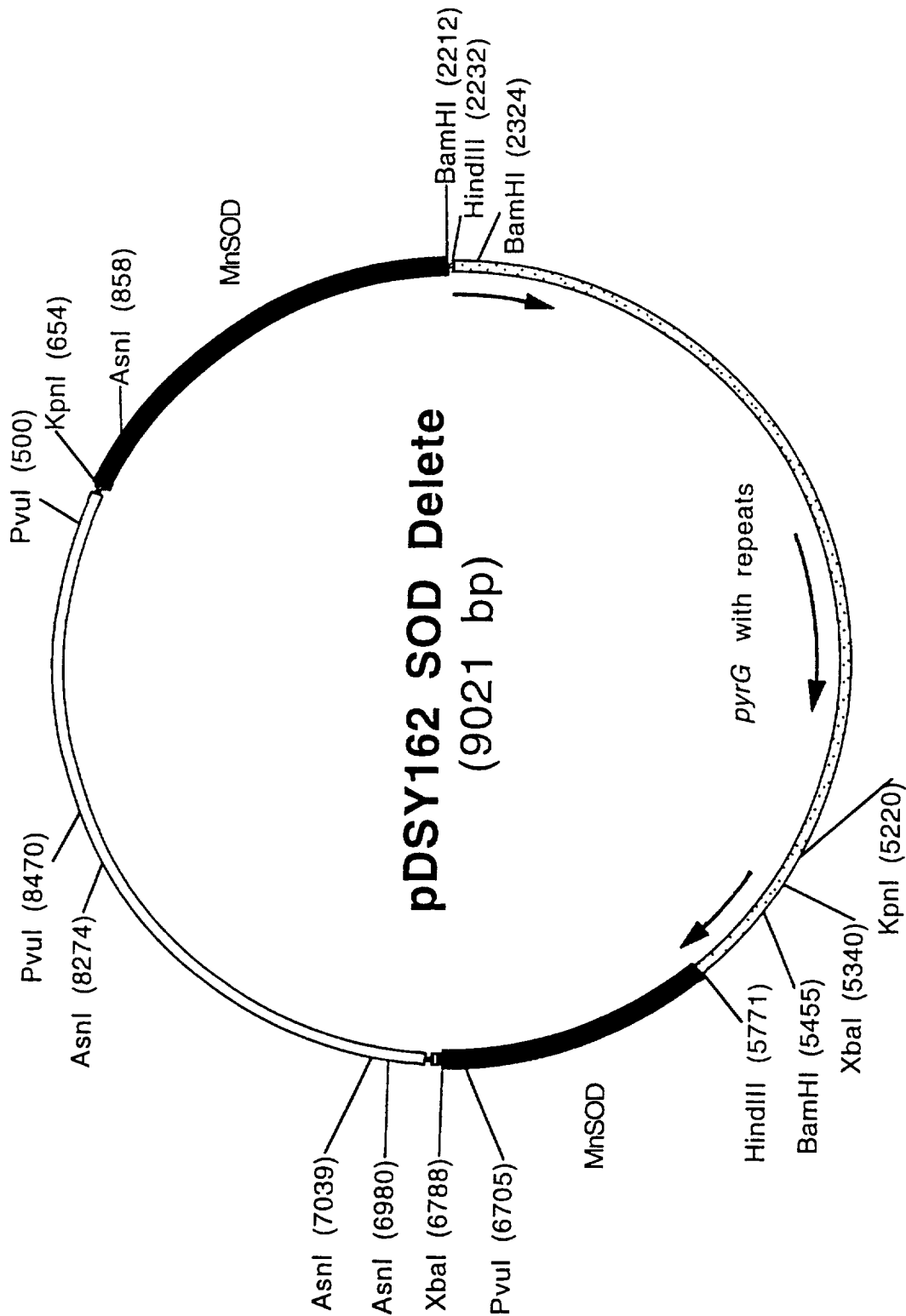


Fig. 19.

ATATCGACTGTAGTAATATCTCAGGTCCTCTGGGATAGCTGATGGAATGTTAAGTGAATAATATTGATTTA
AAGTTCCTCTAGITCCAGCTCTTATGTAGCTTCATTTCTATATATATATCTATTTAGTGGGTGTG
CAGGCGGTGAGCCTATCGGCCAATCATAGTAAAACCCGTTAGTTGCAATACCCCTGTTAGTTGCCAAGGC
GAATTCCTGGCTGATATCCTTGCAACTAACGGCGTTCTCAGTACTCGAATTGAATATATATTTGGCACA
AAGTTATTTGCCAACTTGGGGGCGCTGGGGTCATACAACCCAGCCACCAAGCCTTTATTTAATTOG

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Fig. 20

GATTTAATGACTACCTTGATGATGATACTGCCAATATAGTTAGATAATACAAATCTGGCTGCCATATAAGGC
OCTGGCAACGACATCTTGTTCTTATTNIOOCTCAATCGAGCTTGGCTATGCCCAAGCTTGGAACTATAC
GAGCATTGTAAATTGATTTTGATACGGGCTGCCATATCAGATTGACTC

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Fig. 21

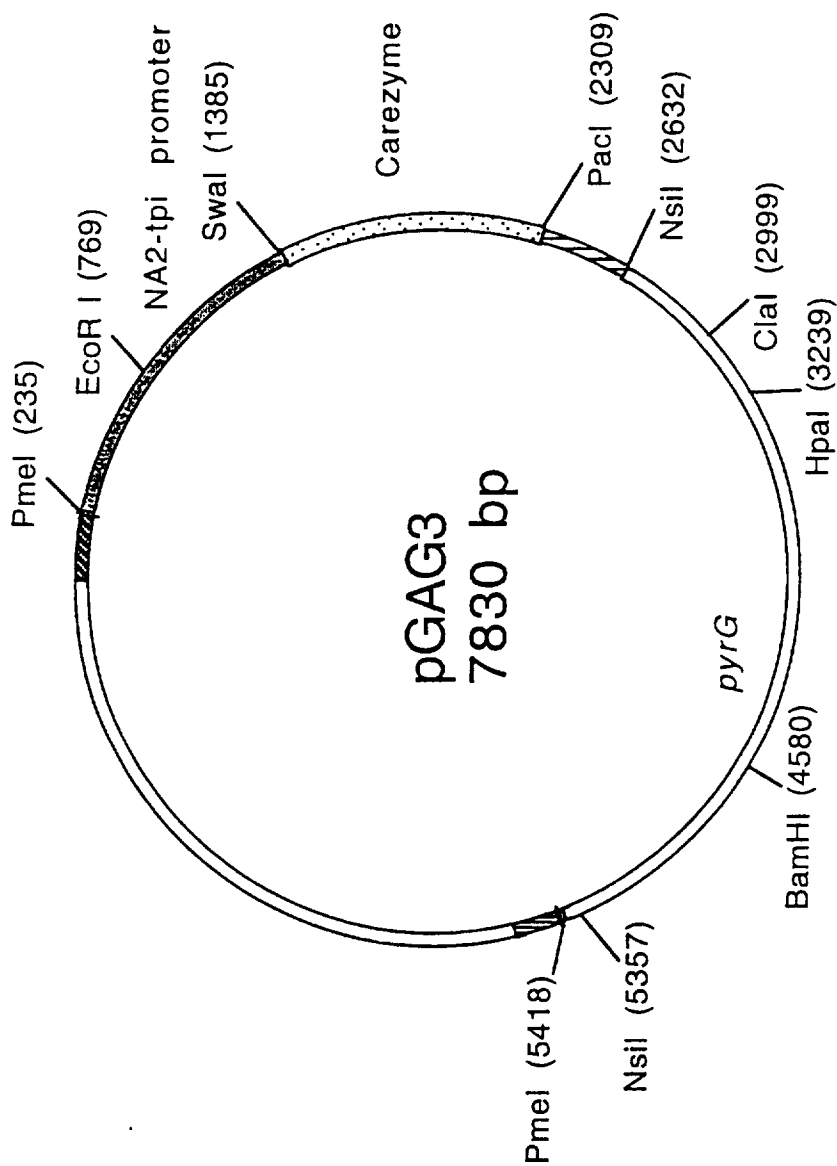


Fig. 22

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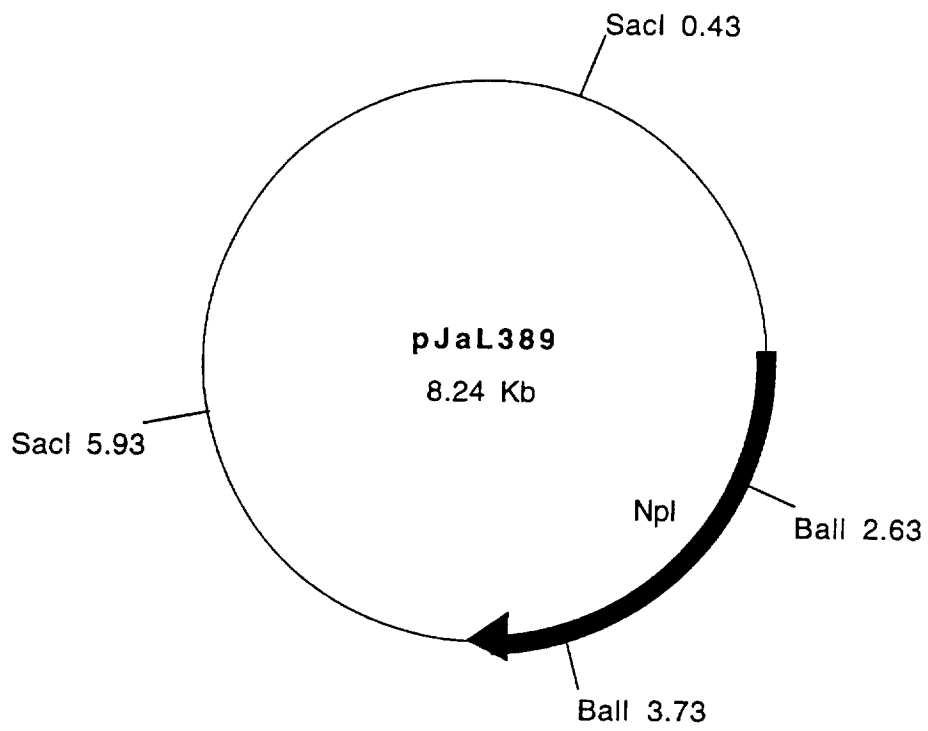


Fig. 23

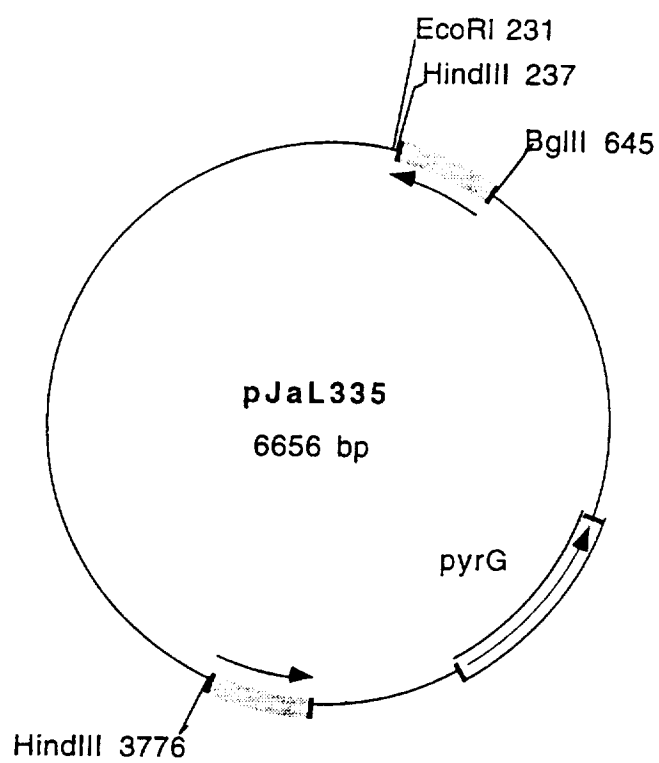


Fig. 24

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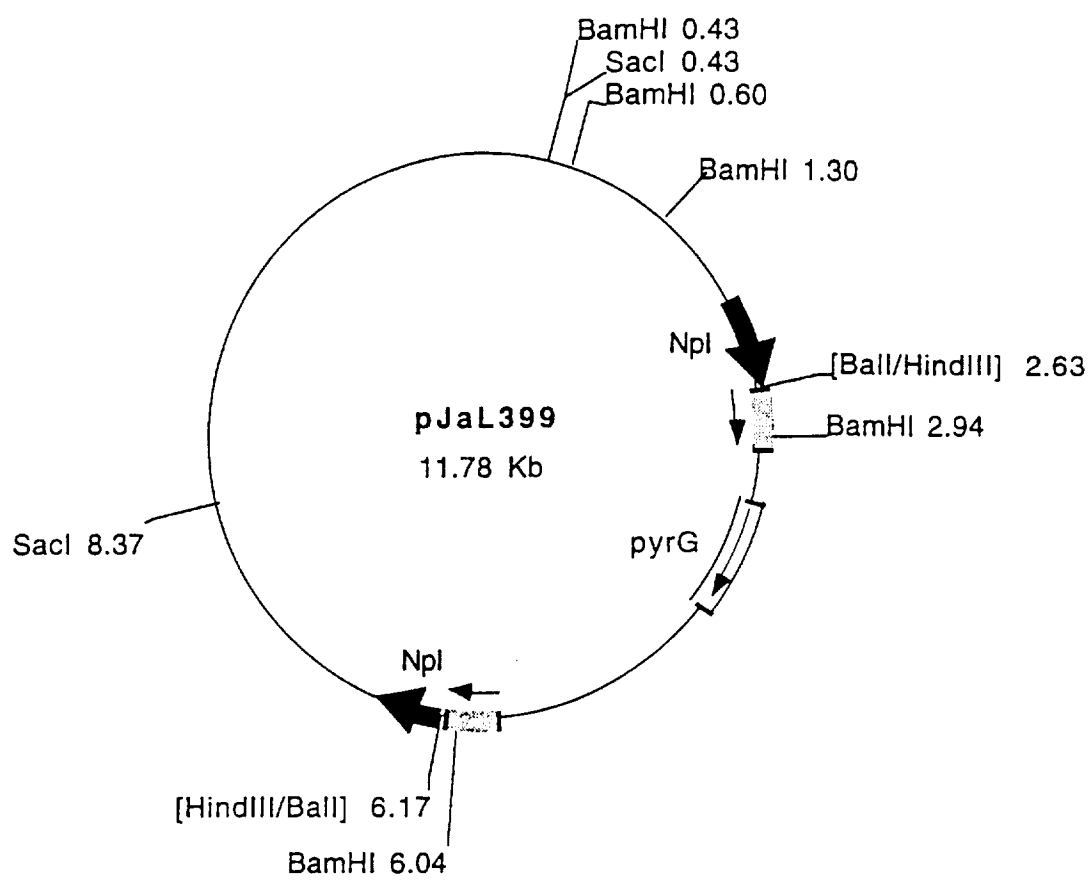


Fig. 25

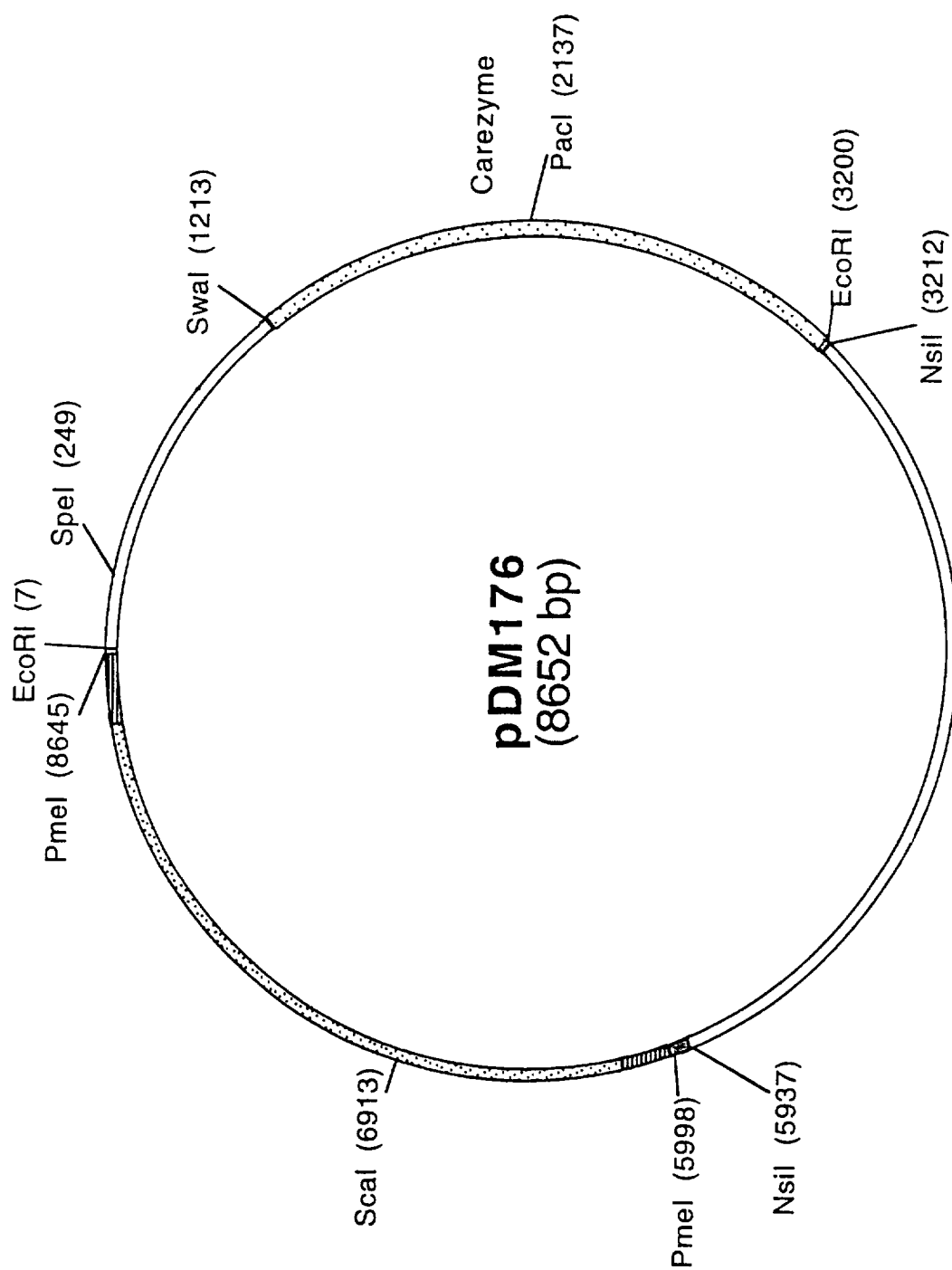


Fig. 26

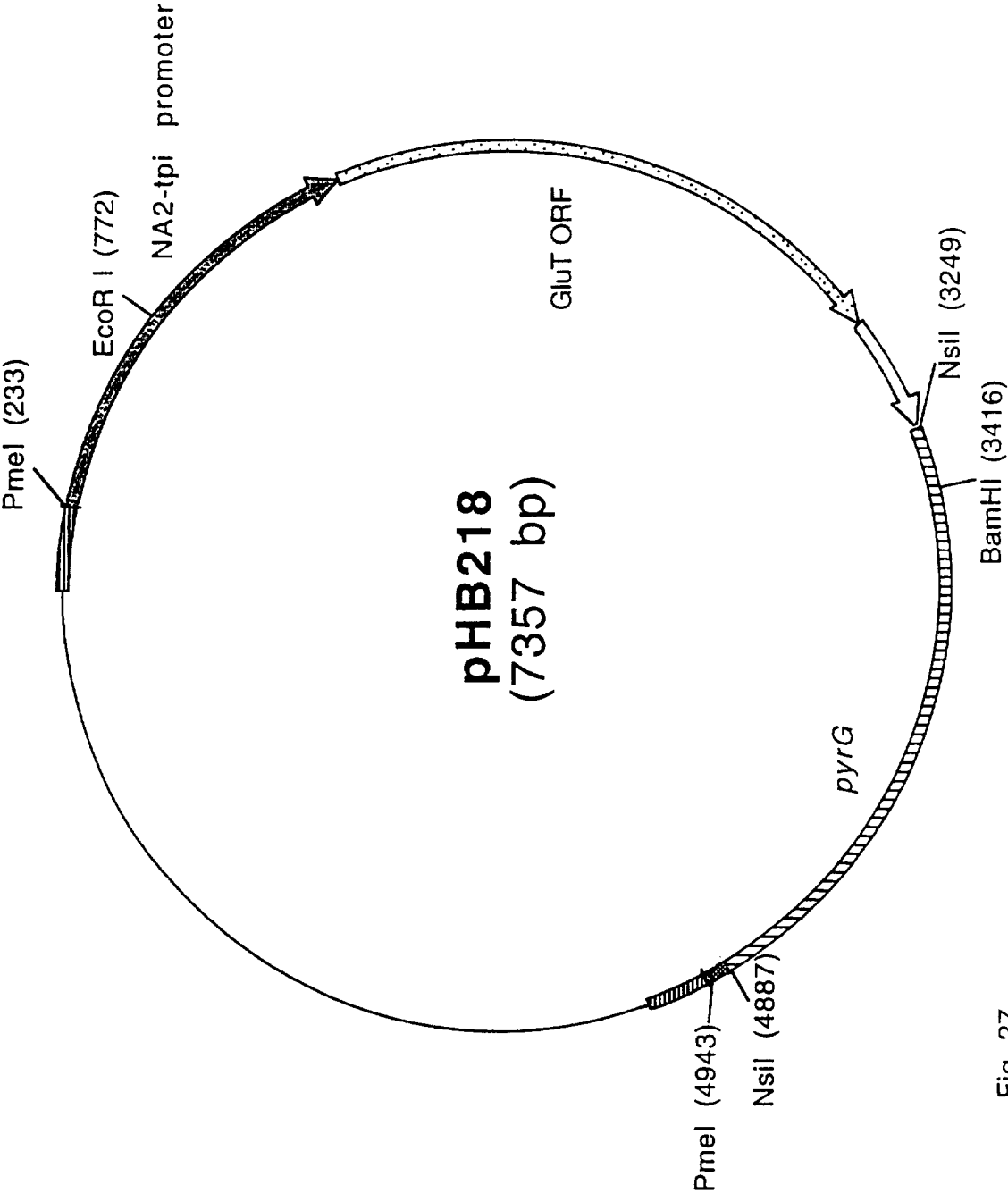


Fig. 27

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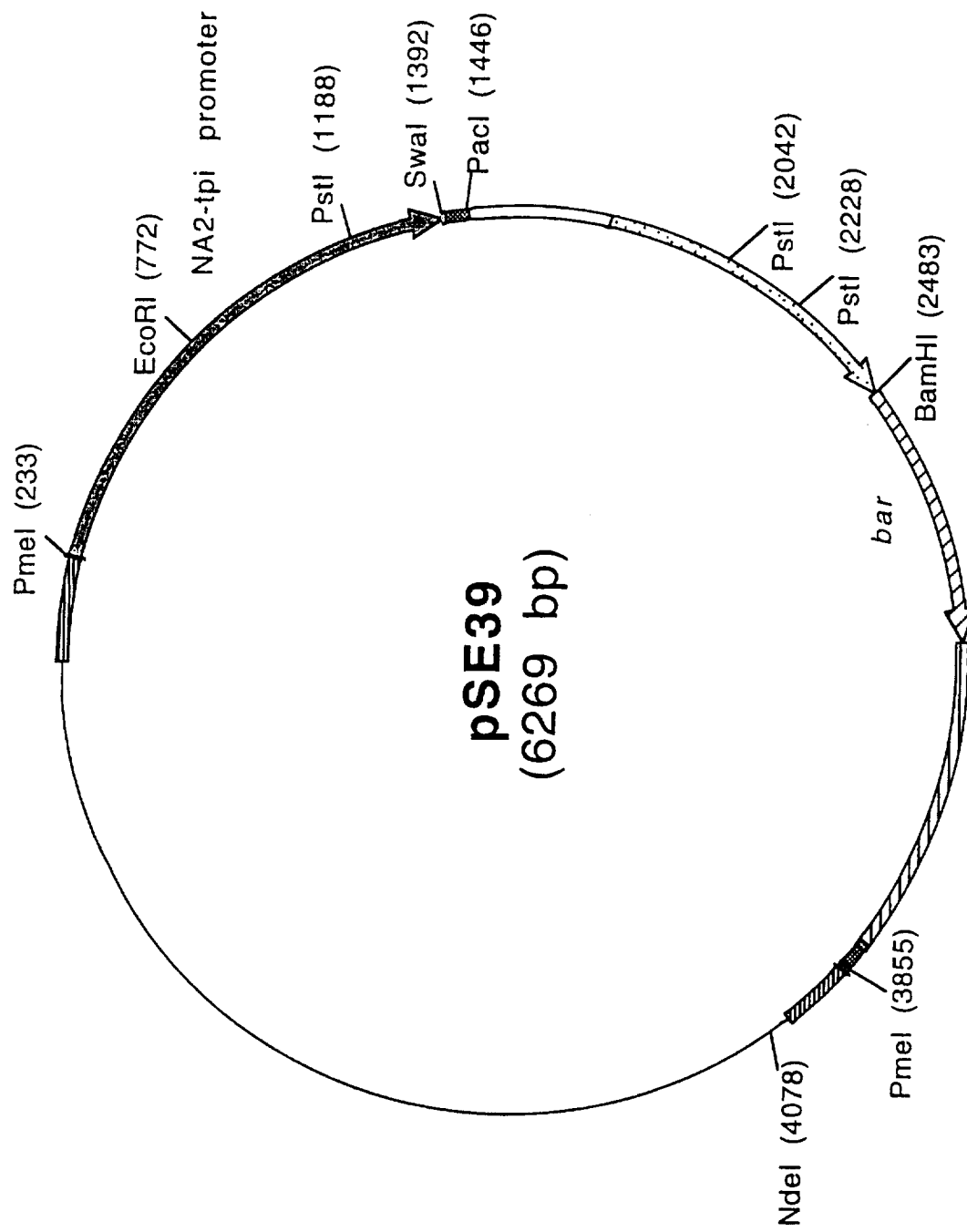


Fig. 28

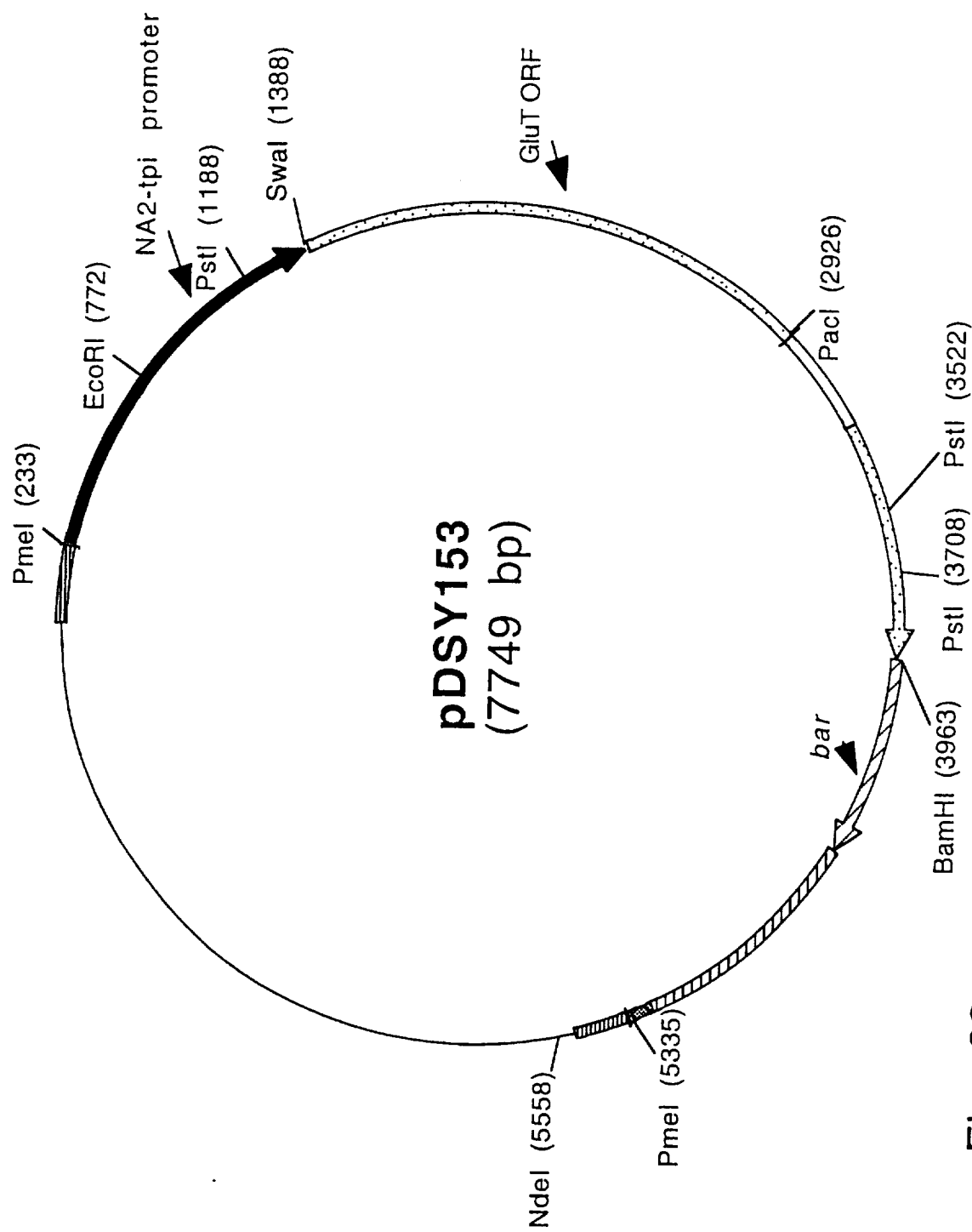
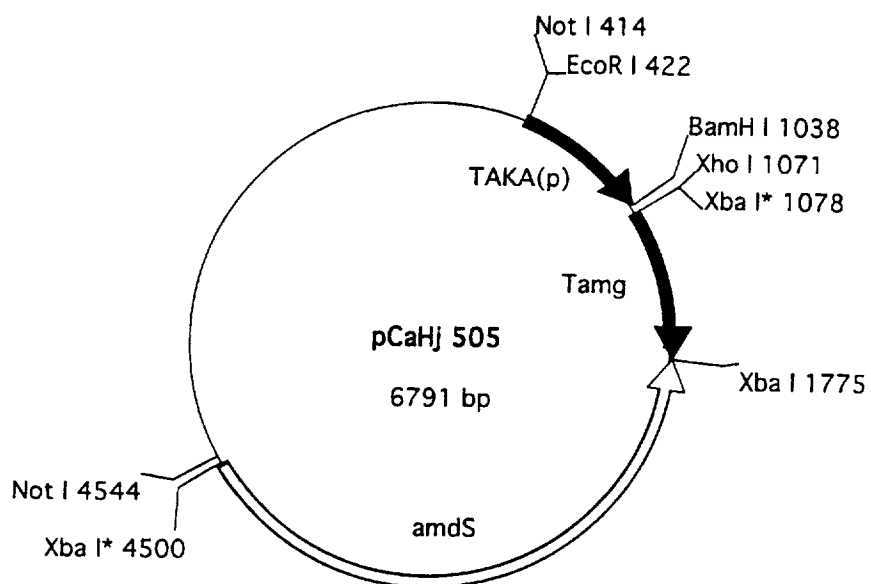


Fig. 29

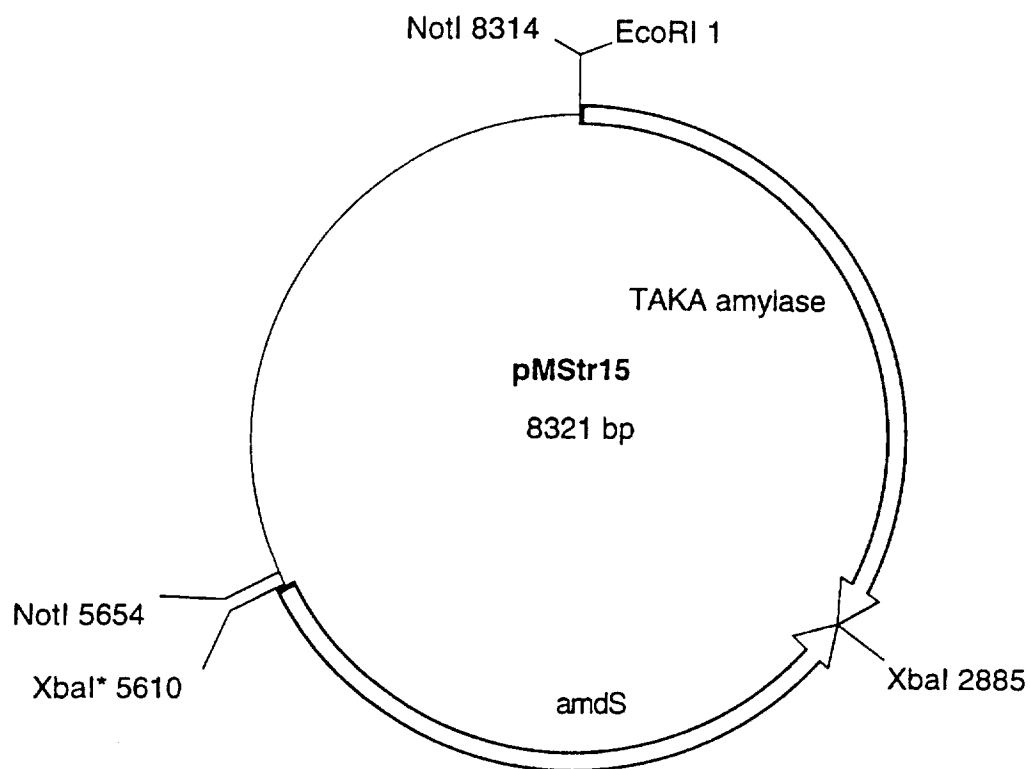
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* methylated sites

Fig. 30

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* methylated site

Fig. 31

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AACCCTGTATGTGAGCCTGATTCAAGACTTCGGATTTACTTTCAAGGCTTCGAATCACTGTTAAGGCAG 70
NPVCEPDSRLRIYFQGFESLLRO
AGAAAGTAGTACTAATGGTTATCAATATATAGGAGGGCAAGCGGGAGGTTATCGCCAACGAAGAAGGA 140
KKVVLMVINI.EGKREVIANE EGG
TTAGTCCACTACTGCTTGGTGGCGGGTAATCTTAAGAGCACAACTAACGGATACACAGATCGTCAAAT 210
.STTAWWRVILRAOTNGYTDROI
CCTTCCGTCCTTTCATACATTGATGGTGAGGAGTACCACGGTACTCAAGCCAAGGCCAGTTGGTCCGC 280
PSVLSYIDGEEYHGTOAKAQLVR
ACTCCCAGAACACTGTCGCATACTTCAGAGATTACCTTGGCAAGGAGTTCAAGTCGATAGACGCCACA 350
NSQNTVAYFRDYLGKEFKSIDATP
ATGCCATAACTCGGCCCATCCTCAGCCTCAGGAGTCTACCGTTGCTTTCTCCATTGTGGACTCTACCAA 420
CHNSAHPQPHESTVAFSIVDSTN
GAGACCCCGAGCACTGTACCGTCTCCGAGATTGCCACCCGCCATCTCCGTCGTTTGAAGCAGTCCGC 490
ETPSTVTVSEIATRHLRLKQSA
CTGACTACCTGGGCAAGGAAGTCAATGCCGCCGTCACTACTGTCCCCACTGACTTCTCCGATGCTCAG 560
SDYLGKEVNAAVITVPTDFSDAQR
CGAGGCTTTGACCGCTTCCGCTAAGGCTGCTGGCCTTGAGGTCTCCAGCTCATCCATGAGCCTGTTGC 630
EALTASAKAAGLEVLIHEPVA
GCTGCCCTGGCTTACGATGCCAGGCCGAGGCTACTGTTACTGACAAGCTTGTTGTCGTCGCCGACCTC 700
AALAYDARPEATVTDKLVVVADL
GTGGTACCCGATCCGACGCTGCTGTTCTCGCTTGCCGTGGTGGCATGTACAGTATCCTCGCAACTGCTC 770
GGTRSDAAVLACRGGMYSILATAH
TGACTACGAGTTGGGTGGAGCTTCGTTGGACAAGATCATCATCGACCATTTCGCCAAGGAGTTCATTAA 840
DYELGGASLDKIIIDHFAKEFIK
AAGCACAAGACCGATCCTCGCGAGAACGCTCGTGGTCTCGCCAAGTTGAAGCTTGAGGGTGAGGCTGC 910
KHKTDPRENARGLA K L K L E G E A A
GCAAGACCTTGAGCTTGGGTACCAACGCCAGCTTGAGCATTGAGATCTTCGCAGATGGCATTGATTTTCG 980
RKTLSLGTNASLSIEIFADGIDFG
CTCCACTGTCAACCGTACTCGNTACGAACTTCTTTCCGGCAAGACCTTCGCCAGTTCACCGGCTTGAT 1050
STVNRTRYELLSGKTFAOFTGLI
GAGCAGGTTATCCAGAAGGCTGGTTTGGATGTTTTGGACATTGACGAGGTTAGTCCCTTGTGATTTTTTT 1120
EQVIQKAGLDVLDIDEVSPL.FF
TTTTTTTTCAG
FFFS 1132

Fig. 32

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70 ATTTCCAGCGTGGTGGTGAACGGTCTCTTTGGGGGGGCTCTATTGGTCATGCATTTGGGTC
 I S Q R R R W L N G S F A A G L Y S L M H F G
 140 GGATGTACAAGGTGGACATAACATCATCGGTATGTTCTTGGACATTCAGATGTTGTACAAGTTTT
 R M Y K S G H N I I R M F F L H I Q M L Y N V F
 210 CAACACTATCGTTACATGGTCTCGCTGGCATCTTACTGGTGAACACACCGTCATCGGACTTGGTC
 N T I L T W F S L A S Y W L T T T V I M D L V
 280 GGAAAGCCCGAGTGAAGCAACGGTAACAAGGATTCCTGGTAAATGGGACCCCTATTATCAACA
 G T P S E S N G N K G F P F G K S A T P I I N
 350 CAATTGTGAAGTATGTCACCTGGATTGTTGCTCTTGCAGTTCATTCGCTCTCGTAAACGGCCAA
 T I V K Y V Y L G L L L Q F I L A L G N R P K
 420 GGGATCGCGTCTCGTAACCTGACATCTTGGTGGTATGGTATCATTCAAATCTACGTTGTGGTAC
 G S R F S Y L T S F V V F G I I Q I Y V V V D
 490 GCTCTGTACTTGGTGGTGGTTCACAAACAGTATGGATAGATTTGGTCAACGATCAAGGTGTG
 A L Y L V V R A F T N S D A I D F V T D Q G V
 535 GCGAATTCCTCAAGTGGTCTCTCTGGTCTTGGGGGGCAGGGCA
 G E F L K S F F S S S G A S A

Fig. 33

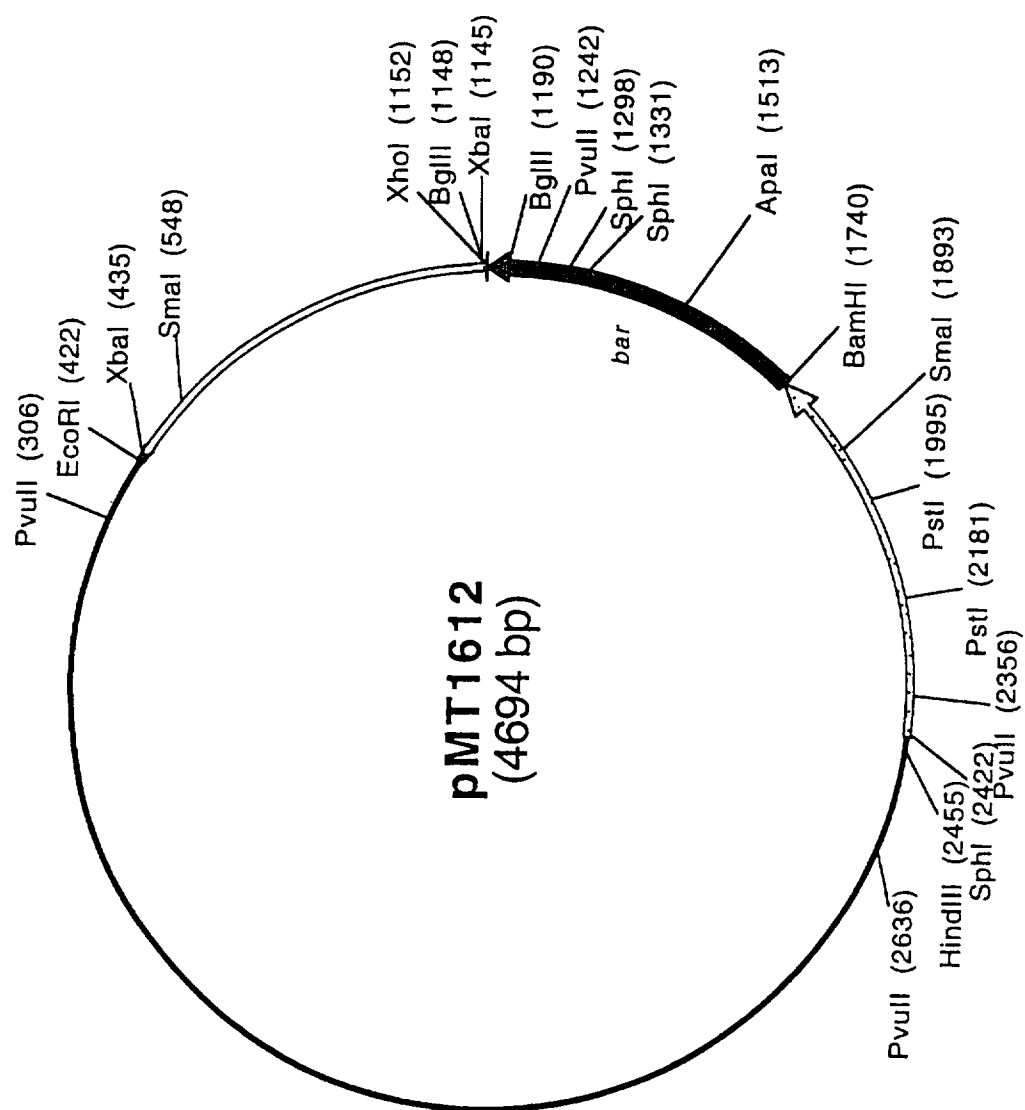


Fig. 34

70	AGGCGCACAGGNTGTGCTAAGGCGCTTGAOCCGGACCOCTGGGACTTTCAAGTCTOCTTGGACOOCCGAATT
140	G P Q ? V L S A L T G T L G L S S L L G P G I GAATCCTCACAGACAGCTTTCAACACTGCTCTAAGGCTGAAGTGAAGTGGGACTOOGTATCATGCGC
210	E S S Q N S F Q H C S K A E L S C A T P Y H G AAGACAAATGCTGCTTCAACTATOOCCGGGGGAGTTCCTTCAATGCTGTTTGGGACGCGCGAOCOCGC
251	Q D K C C F N Y P G G Q F L Q S L F W D A D P A CATTGGAOCGGAAGATTCTGGACTATOCATGCTTATGGT I G P E D S W T I H G L W

Fig. 35

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CTACTGAAGCCTTAAGGTGCTTAAGGAGCAACTTCATATTATGGCGAOC CAAGGATOCAGGAAGTCIT
GAGCAATAGCAAGGGTGGAAAGGAGCAAGGAGCACTGCGACAGCGAOCAGGTTTGGGEGACTCAAAGG
TCTGGCTAAAGGAGGCTTTGTGGAGGTGGAATGCGGAAGAATTCCAAAGCAATTGGCGAATTGGCCA
CCCC

Fig. 36

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AGCAOCTATAATCTATGCTGTGCCACTATCACACATCTATATGTTGTACAAGCCTGATACAATCAATAAT
GATGTAAATAATTGACTCTGGAAAGTTGGCTATAAAACTCACCATACAAGTCCAGATAAOCTGCCAAACT
OACTOCCAGGECATTAACTTCAITTTATATOGACCAGCCATAOCTATGGTCAAAATCACACGCAAGGCCA
CAGATATATATTTGAATCAAAATTTCTCTTTTGAAGAAGAAAGGTGGTTTATGAGGAAGAATATOOCAAT
ATGCCAATCTGACTGTTCCGGATTGGAATAATGCACAAGCATTGCTOCTCTTTATAGAGAAACTGACAGGA
CAAGACCTAOCOCATGACATATAATTGGAATATAAATATA

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Fig. 37

CAAGCGAOCGGGAATAAAATACACATACAAAGGATTGGAAGAAAGAAAAAAGCGGCGAGGTGTGT
CCAAGAGCGAAGAGAAAAAAATTTAATTTGGCCACCCCTATGGCGAGTGTTCGGCCTTCAGGAGAGAT
AGAAAAGAGCGGAGAGCGAGAGGAAAAAAACAGAAATTOCCACAGACAAGGAAAGCTTAACCG
GGTCACGAAAAAGCACAATACAGGTGAACAACCTGAGGCGAAGCGGCGCAAAAGAAAAAATATATTOCTA
A

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Fig. 38

INTERNATIONAL SEARCH REPORT

International Application No
PCT/US 97/16604

A. CLASSIFICATION OF SUBJECT MATTER

IPC 6 C12N5/10 C12P21/00

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC 6 C12N C12P

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 94 13813 A (RHONE POULENC BIOCHIMIE) 23 June 1994 cited in the application see the whole document ---	1-21,23, 26,38, 40-42, 52-54
X	WO 92 17594 A (UPJOHN CO) 15 October 1992 see the whole document --- -/--	1,2,4,6, 7,9,11, 12,14, 16,17, 19,26, 31,32, 38,42, 44,53,54

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Date of the actual completion of the international search

5 December 1997

Date of mailing of the international search report

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Chakravarty, A

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PCT/US 97/16604

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Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	WO 96 09397 A (NOVO NORDISK BIOTECH INC) 28 March 1996 see the whole document ---	1-54
Y	WO 92 10561 A (UNIV FLORIDA) 25 June 1992 see the whole document ---	1-54
Y	EP 0 338 410 A (CONSORTIUM ELEKTROCHEM IND GMBH) 25 October 1989 see page 2, line 42 - page 3, line 56 ---	1-54
P,X	WO 96 29414 A (GENENCOR INT INC) 26 September 1996 cited in the application see the whole document -----	1-54

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Information on patent family members

Intern. Appl. No.

PCT/US 97/16604

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9413813 A	23-06-94	FR 2698879 A CA 2151123 A EP 0673422 A JP 8505286 T	10-06-94 23-06-94 27-09-95 11-06-96
WO 9217594 A	15-10-92	AT 123527 T AU 1753092 A DE 69202859 D DE 69202859 T EP 0578754 A ES 2074887 T JP 6506118 T	15-06-95 02-11-92 13-07-95 07-12-95 19-01-94 16-09-95 14-07-94
WO 9609397 A	28-03-96	US 5594119 A AU 3593195 A CN 1137293 A EP 0734444 A FI 962099 A JP 9501061 T US 5688663 A ZA 9507883 A	14-01-97 09-04-96 04-12-96 02-10-96 17-07-96 04-02-97 18-11-97 20-03-96
WO 9210561 A	25-06-92	AU 6194696 A AU 668677 B AU 9098891 A EP 0560885 A JP 6504436 T US 5482846 A	31-10-96 16-05-96 08-07-92 22-09-93 26-05-94 09-01-96
EP 0338410 A	25-10-89	DE 3813107 A AT 112320 T DE 58908428 D DK 186589 A JP 2035077 A	02-11-89 15-10-94 03-11-94 20-10-89 05-02-90
WO 9629414 A	26-09-96	NONE	