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(72) Inventeur/Inventor:
STUART, W. DORSEY, US

(73) Propriétaire/Owner:
UNIVERSITY OF HAWAII, US

(74) Agent: FETHERSTONHAUGH & CO.

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(71) Applicant: UNIVERSITY OF HAWAII [US/US]; Manoa Innovation Center, 2800 Woodlawn Drive, #280, Honolulu, HI 96822 (US).

(72) Inventor: STUART, W., Dorsey; 46-369 Haiku Road, H-9, Kaneohe, HI 96744 (US).

(74) Agents: MURASHIGE, Kate, H. et al.; Morrison & Foerster, 2000 Pennsylvania Avenue, N.W., Washington, DC 20006 (US).

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(57) Abstract

The invention relates to a heterokaryotic filamentous fungus host capable of producing a heterologous heterodimer comprising at least two subunits. The heterokaryon contains a first and second nucleus; each nucleus contains an expression system for one subunit of the heterodimer. The heterokaryon is prepared by culturing together a first fungus host strain and a second fungus host strain that is homozygous with the first fungus host strain with respect to all heterokaryon compatibility alleles, wherein the first and second fungus host strains are cultured together under conditions wherein neither the first nor the second fungus strain can survive unless the heterokaryotic host is formed.

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HETEROLOGOUS DIMERIC PROTEINS PRODUCED IN HETEROKARYONS

Field of the Invention

The present invention relates generally to the expression of heterologous genes in filamentous fungi, more specifically to the expression of genes encoding heterodimeric proteins by heterokaryotic filamentous fungi host cells.

Description of the Related Art

The cloning and expression of heterologous genes in bacteria, yeast and fungi have been recognized as a viable means for producing a variety of useful proteins. Expression of heterologous genes in these microorganisms has generally relied on the use of autonomously replicating extrachromosomal elements, widely known as plasmids. For example, Lambowitz, U.S. Patent No. 4,486,533 issued December 4, 1984, discloses the autonomous replication of DNA vectors for filamentous fungi by mitochondrial plasmid DNA. The mitochondrial plasmid DNA may be joined to another replicating system to provide a shuttle vector to enhance the convenience of genetic manipulation. Yelton *et al.*, U.S. Patent No. 4,816,405 issued March 28, 1989, describes tools and systems that enable the modification of important strains of filamentous ascomycetes to produce and secrete large quantities of desired heterologous proteins

Buxton *et al.*, U.S. Patent No. 4,885,249 issued December 5, 1989, discloses the transformation of *Aspergillus niger* by a DNA vector that contains a selectable marker capable of being incorporated into the host *A. niger* cells. The vector may also contain other foreign DNA sequences required to enhance or modify the expression of proteins. McKnight *et al.*, U.S. Patent No. 4,935,349 issued June 19, 1990, discloses a method for expressing higher eukaryotic genes in *Aspergillus* involving promoters capable of directing the expression of a heterologous gene in *Aspergillus* and other filamentous fungi. Similar techniques have been used to clone the mtr gene involved with amino acid transport in *Neurospora crassa* ("*N. crassa*") and to

verify the tight linking of the cloned DNA to genomic markers flanking this gene *in vivo*. Stuart, W.D. *et al.*, Genome (1988) 30:198-203; Koo, K. and Stuart, W.D. Genome (1991) 34:644-651.

However, production of a heterologous, dimeric protein, which has two or more non-identical subunits, in a fungal host cell has required the transformation of a single host cell in one of the following two ways:

(1) by a single large, unwieldy vector carrying the sequences for both subunits; or

(2) by two smaller separate vectors, each of which carries a DNA sequence encoding one of the subunits, on the assumption that at least some portion of the transformed cells will be capable of carrying both subunits sufficiently close together, spatially and functionally, to enable the simultaneous expression of both genes.

Burke *et al.*, U.S. Patent No. 4,880,734 issued November 14, 1989, discloses DNA constructs having a transcription control region comprising two regions, a first transcriptional regulatory region and a second transcriptional initiation region, where the two regions may be derived from different sources. This two-part transcriptional control region was joined to a gene not naturally associated with the transcriptional control region. A terminator region was also present to provide an expression construct that can be introduced into a yeast host as a extrachromosomal element. The use of regulatory sequences for controlling transcription of a structural gene provided the ability to grow the host cells to a high density with little or no expression of the structural gene, and then to induce expression by changing the environmental conditions, e.g., metabolites, temperature, etc.

European Patent No. 552,569 published July 28, 1993 discloses a method of fusing (a) an animal cell capable of proliferating in a basal medium and (b) an animal cell having the ability to produce a useful substance and the ability to proliferate in a complete medium, but not in the basal medium. The resulting fused cell has both the ability to produce the

useful substance and to proliferate in the basal medium. (EP 552,569, column 1, lines 45-53.)

There remains in the art a need to produce a heterologous heterodimer in a reliable and efficient manner. None of the above disclosures provides a method to do so.

Disclosure of the Invention

The invention is directed to a heterokaryotic filamentous fungus capable of producing a heterologous heterodimer comprising at least two non-identical subunits. The heterokaryotic fungus is the result of fusion of two complementary fungal strains. The fusion results because each of the parent strains supplies a requirement of the other under the conditions of culturing.

Thus, in one aspect, the invention is directed a method to prepare a heterokaryotic filamentous fungus containing a first nucleus which has been modified to contain an expression system for a first nucleotide sequence encoding one subunit of a heterologous heterodimer as well as a second nucleus modified to contain an expression system for the production of the other subunit of the heterodimer. The process involves fusing separate strains of the fungus, one containing the first nucleus and the other the second. The fusion results because the first nucleus also confers a first characteristic negatively affecting growth under specified conditions that is correctable by a first property conferred by the second nucleus, and conversely the second nucleus also confers a second characteristic negatively affecting growth under specified conditions that is correctable by a second property conferred by the first nucleus. Thus, the property conferred by each nucleus complements the characteristic confined by the other when the conditions are such that both properties are required for growth. In a simple example, each nucleus may contain a mutant genome which results in an inability to grow in the absence of a different nutrient. Fungi containing both nuclei, but not those containing only one, will be able to grow in the absence of both nutrients.

This aspect of the invention is thus a method to prepare the heterokaryotic fungus of the invention by culturing

together the above first and second fungus strains under conditions that, due to the presence of the first and second characteristics of the nuclei, neither the first fungus nor the second fungus host strain can survive unless the heterokaryotic fungus is formed. The resulting heterokaryotic filamentous fungus can then be kept in a heterokaryotic state by maintaining the fused fungus in a culture medium under these same conditions.

When cultured under these same conditions to maintain the heterokaryotic state, the desired heterodimer protein can be recovered when the conditions also include those favorable for the expression of the nucleotide sequences encoding the subunits therefor. Thus, another aspect of the invention is production of the heterologous dimer by culturing the heterokaryotic fungus under these conditions and recovering the heterodimer.

In still other aspects, the invention is directed to a filamentous fungus heterokaryon comprising at least two nuclei, one of which contains an expression system for production of one subunit of a heterologous heterodimer and the other which contains an expression system for production of the other subunit of the heterodimer.

Various embodiments of this invention provide a heterokaryon filamentous fungus which fungus contains a first nucleus and a second nucleus, wherein said first nucleus has been modified to contain an expression system for a first nucleotide sequence encoding a first subunit of a heterologous heterodimer and said second nucleus has been modified to contain an expression system for a second nucleotide sequence encoding a second subunit of said heterologous heterodimer.

Other embodiments of this invention provide a method to prepare the aforementioned heterokaryon fungus of this invention, which method comprises: culturing a first fungus containing said first nucleus, wherein said first nucleus confers a first characteristic negatively affecting growth under specified conditions that is correctable by a first property conferred by the second nucleus, along with a second fungus containing said second nucleus wherein said second nucleus confers a second characteristic negatively affecting growth under said specified conditions that is correctable by a second property conferred by the first nucleus; said culturing being conducted under said specified conditions.

Various other embodiments of this invention provide a method to produce a heterodimer heterologous to a filamentous fungus, which method comprises culturing a heterokaryon filamentous fungus of this invention under conditions wherein said first and second subunits are produced to form said heterodimer; and recovering the heterodimer from the culture.

Brief Description of the Drawings

Figure 1 shows the nucleotide sequence of an approximately 2.9 kb fragment of the *N. crassa mtr* gene containing the entire open reading frame as well as the promoter and transcription terminating signals.

Figure 2 shows nucleotide and deduced amino acid sequences of the open reading frame of the *mtr* locus.

Modes of Carrying Out Invention

In the present invention, advantage is taken of the ability of filamentous fungi to form heterokaryons; the heterokaryons can then be used to produce heterologous heterodimers.

Nature of Filamentous Fungi and Background Requirements for Heterokaryon Formation

Fungi can occur in single mononucleated cells that yield filamentous multinuclear strands, yeast cells, fruiting bodies with diverse spores, and/or cells that are differentiated sexually. They can also exist in multinucleated forms. The principal element of the growing form of a fungus as a mold is the hypha, a branching tubular structure, about 2μm-10μm in diameter. Hyphae grow by elongation at their tips (apical growth) and by producing side branches. Thus, as a colony grows, its hyphae form a mass of intertwining strands.

Some hyphae penetrate into the culture medium on which the fungus is growing to absorb nutrients, while those hyphae that project above the surface of the medium constitute an "aerial mycelium." Most colonies grow at the surface of liquid or solid media as irregular, dry, filamentous mats. In most species, the hyphae are divided by cross-walls called "septa." These septa, however, have fine, central pores. Thus, even septate hyphae have nuclei that are embedded in a continuous mass of cytoplasm and, in effect, contain a multiplicity of nuclei in a transportable cytoplasm.

The term "filamentous fungi" refers to those fungi that can form a mycelium through a mass of branching, interlocking filaments and, although interrupted by cross walls, permit the passage of cytoplasm between compartments due to perforations in the cross walls. Many of these fungi form meiotic spores within a sac when propagated sexually. With the appropriate stimulation, however, the mechanism of which is not entirely understood, reproduction can occur asexually. In this manner of reproduction, spores known as "conidia" are borne externally at the tips of budding projections formed at various locations along the filaments.

The filamentous fungi of the invention are generally *Phycomycetes*, *Ascomycetes*, *Basidiomycetes*, and *Deuteromycetes*. The *Phycomycetes* include all non-septate, as well as some septate, filamentous fungi. Their asexual spores are of various kinds and include sporangiospores contained within sacs formed

at the end of specialized stalks. Different species have different sexual cycles.

Ascomycetes are distinguished from other fungi by the ascus, a saclike structure containing sexual spores, known as ascospores. The ascospores are the end product of mating, the fusion of male and female nuclei, two meiotic divisions, and usually one final mitotic division. *Basidiomycetes* are distinguished by sexual spores that form on the surface of a specialized structure. The *Deuteromycetes* are often referred to as "imperfect fungi" because no sexual phase has yet been observed. Their hyphae are septate, and conidial forms are similar to those of the *Ascomycetes*.

The preferred heterokaryotic filamentous fungus is of the group Ascomycetes, more preferably, from the genera *Neurospora*, *Aspergillus* and *Penicillium*. Particularly useful species from *Neurospora* include *N. intermedia*, *N. crassa*, *N. sitopula*, and *N. tetraspora*.

Useful species of *Aspergillus* include *A. nidulans*, *A. niger*, *A. terreus*, and *A. fumegatus*.

A particularly preferred genus is *Neurospora*, of which the most preferred species is *N. crassa*.

The vegetative growth of filamentous fungi involves nuclear division with cell division (mitosis). This type of cell division consists of asexual reproduction, i.e., the formation of a new clone without the involvement of gametes and without nuclear fusion by way of conidia. For example, the species of *Neurospora* contain in their nuclei seven different chromosomes, each having a single copy, i.e., the vegetative organism is haploid. This haploid state is typically maintained during mycelial growth and during asexual reproduction through the formation of conidia.

Sexual reproduction can also occur, and then two haploid cells (hyphae or conidia) of different mating type fuse to form a dikaryotic cell containing two distinct nuclei. The two haploid nuclei thus coexist in the same cytoplasm and, for a time, divide more or less in synchrony. If a cell initiates ascospore formation, however, the two different haploid nuclei can actually fuse to form a diploid nucleus, which contains

pairs of homologous chromosomes. This diploid cell then begins meiosis.

A "heterokaryon" is a cell with two (or more) genetically different nuclei. The heterokaryons of the invention must contain nuclei from cells that are homozygous for all heterokaryon compatibility alleles (except for the mating type allele when the tol gene is present). At least ten chromosomal loci have been identified for heterokaryon incompatibility: het-c, het-d, het-e, het-i, het-5, het-6, het-7, het-8, het-9 and het-10, and more are inferred to exist. Perkins *et al.*, "Chromosomal Loci of *Neurospora crassa*", Microbiological Reviews (1982) 46:426-570, at 478.

If two strains carry different alleles at one or more het loci, they are unable to form stable heterokaryons. Protoplasmic killing occurs after fusion of unlike hyphae or after microinjection of cytoplasm or extracts into unlike strains. When duplications (partial diploids) are heterozygous for het one or more alleles, growth is inhibited and highly abnormal. A number of heterokaryon incompatibility loci (specifically, het-c, -d, -e, and -i) were first defined by heterokaryon tests. Het-5 through -10 loci were detected by using duplications, as differences at het loci are common in natural populations. Id.

Mating type alleles "A" and "a" also act as het genes in *N. crassa*, although some slow heterokaryotic growth may occur. Microinjection experiments have implicated proteins in the killing reaction. Thus, opposite mating types are also generally important for the complex events associated with the proliferation of heterokaryotic ascogenous hyphae. Id. at 436 and 478. However, if the tol gene is present, the vegetative (heterokaryon) incompatibility associated with opposite mating type alleles A and a is suppressed without sexual compatibility being affected. Thus, (tol; A + a; a) heterokaryons can be fully compatible and stable if the other het loci are homokaryotic and A/a duplications grow normally when the tol gene is present.

If hyphae from two different strains that are homozygous for the compatibility loci are provided, they may

fuse when grown in the same medium, in particular when fusion is forced as described below. The resulting culture will then contain nuclei from both strains circulating in the shared cytoplasm of a common mycelial mat.

Nature of the Parent Strains

Since each of the parent fungi used in the fusion will provide an expression system for a subunit of a heterodimer, one parent will have a nucleus modified to contain an expression system for a nucleotide sequence encoding a first subunit of a desired heterologous heterodimer and second fungus parent will have a nucleus modified to contain an expression system for a nucleotide sequence encoding the second subunit of the desired heterologous heterodimer, which is different from the first subunit. Transformation of each parent strain with DNA comprising an expression system for the relevant subunit is conducted using standard recombinant techniques, as further described below.

In addition to having been modified to contain the desired expression systems, the nuclei of each of the parent strains must contain a genome that results in a characteristic that renders the fungus dependent on the presence of the second nucleus for survival under the conditions provided for fusion to form the heterokaryon. Thus, the nucleus of each parent confers a characteristic which would result in the failure of the fungus in which it is contained to survive under the culture conditions unless the second nucleus is also present. For example, a parent that requires a particular nutrient may be cultured on a medium lacking the nutrient along with a parent that does not have this requirement. If hyphal fusion occurs, the nucleus of the second parent confers ability to survive in the absence of this nutrient. The second parent, in turn, may require a different nutrient, not required by the first. Only fungi containing both nuclei can then survive when both nutrients are lacking.

The required nutrient can be any substance which the fungus strain cell needs for growth or which, when absent, seriously impairs the ability of the fungus strain to grow or

survive. Examples of useful nutrient requirements and the relevant mutants include:

(1) amino acids such as histidine (his-1 through -7 mutants), proline (aga mutants), arginine (arg-11 mutants), citrulline (arg-11 mutants), asparagine (asn mutants), choline (chol-1 and chol-2 mutants), cysteine (cys-1 mutants), glutamine (gln-1 mutants), leucine (leu-1 through -4), lysine (lys-2, -4 and -5), methionine (mac mutants and met-6, -9 and -10 mutants), and threonine (thr-2 and -3 mutants);

(2) mixtures of aromatic amino acids, such as a mixture of p-aminobenzoic acid, tyrosine, tryptophan, and phenylalanine (required by all aro strains except aro-6, aro-7 and aro-8), a mixture of tryptophan and phenylalanine (required for aro-6 mutants), a mixture of isoleucine and valine (required for ilv-1, -2 and -3), and a mixture of phenylalanine and tyrosine (required for pt mutants);

(3) vitamins such as pantothenic acid (pan-1 mutants) and thiamine (thi-2 and thi-4 mutants);

(4) purine bases such as adenine (ad-2 through ad-4 and ad-8 mutants), hypoxanthine (ad-2 and ad-3 mutants), inosine, and guanine or guanosine (gua-1 or -2 mutants);

(5) pyrimidine bases such as uracil (pyr-1 through pyr-6);

(6) saturated fatty acids (cel mutants) or unsaturated fatty acids such as C_{16} or C_{18} fatty acids having a double bond in the cis conformation at either the 9- or 11-position, fatty acids with a double bond in the trans configuration at the 9-position, and fatty acids with multiple cis double bonds interrupted by methylene bridges (ufa-1 and -2);

(7) physiologically important ions such as potassium (trk);

(8) sugar alcohols such as inositol (acu mutants and inl mutants) and glycerol; and

(9) other organic entities such as acetate (ace mutants), α-ketoglutarate, succinate, malate, formate or formaldehyde (for mutants), p-aminobenzoic acid (pab-1, -2 and -3 mutants), and sulfonamide (sfo mutants at 35°C).

One specific example based on a nutritional requirement is the Arg B+ gene coding for the enzyme ornithine transcarbamylase. This enzyme is present in wild type *A. niger*. Mutants lacking this enzyme (Arg B- strains) can be prepared by usual non-specific techniques, such as treatment with ultraviolet radiation, followed by screening based on an inability to grow on minimal medium, coupled with an ability to grow on a medium containing arginine. Fungi containing this genome will grow on minimal medium if they also include an ArgB+ nucleus.

Also useful for forcing heterokaryon formation are genes conferring a resistance to any one of a variety of cytotoxic agents. For example, in an alternative embodiment, one of the parents can have a requirement for a nutrient as well as a resistance to a toxic effect induced by a noxious chemical, an antibiotic or virus, or a harsh environmental conditions such as a predetermined temperature range to which the other parent is sensitive.

Specific examples of noxious chemicals that can exert a toxic effect include acriflavine (resistance conferred by acr generally, with the presence of the shg gene being required for resistance by acr-4 and acr-6); 3-amino-1,2,4-triazole (resistance conferred by acr-2, atr-1, cpc, leu-1 or leu-2)); dyes such as malachite green (resistance conferred by acr-3); caffeine (resistance conferred by caf-1); purine analogs (resistance to 8-azaadenine and 2,6-diaminopurine conferred by aza-1; resistance to 8-azaadenine and 8-azaguanine conferred by aza-2; resistance to 8-azaguanine and 6-mercaptopurine conferred by aza-3; resistance to 6-methylpurine conferred by mep(3) and mep(10); cyanide (insensitivity conferred by cni-1 in the first 24 hours of growth); tetrazolium (resistance conferred by cya-6 and cya-7); cycloheximide (resistance conferred by cyh-1, -2 and -3); chromate (resistance conferred by cys-13); 2-deoxy-D-glucose (resistance conferred by dgr^-); edeine (resistance conferred by edr-1 and -2); ethionine (resistance conferred by eth-1, by nap in the presence of p-fluorophenylalanine, and by oxD if the ethionine is in the D form); fluoro compounds such as 5-fluorodeoxyuridine, 5-fluorouracil, and 5-fluorouridine

(resistance to all three conferred by fdu-2; resistance to 5-fluorouracil being conferred by uc-5 in an ammonia-free minimal medium; resistance to 5-fluorodeoxyuridine and 5-fluorouridine being conferred by ud-1), and fluorophenylalanine (resistance conferred by fpr-1 through -6 under certain conditions); 8-azaadenine (resistance conferred by mts); methyl methane sulfonate (insensitive or marginally sensitive for upr-1); surface-active agents such as dequalinium chloride, cetyltrimethyl ammonium bromide, and benzalkonium chloride (resistance conferred by sur-1); and metal ions such as vanadate (resistance conferred by van).

Examples of antibiotics typically exerting a toxic effect include benomyl [methyl-1-(butylcarbamolbenzimidazol-2-yl carbamate] (resistance conferred by Bml); antimycin A (insensitivity conferred by cni-1 in the first 24 hours of growth); polyene antibiotics such as nystatin (resistance conferred by erg-1 and -3); and oligomycin (resistance conferred by oli).

Also useful are genes conferring resistance to extremes in various environmental conditions such as a high or low temperature, the lack of oxygen (resistance conferred by an), constant light (resistance conferred by lis-1, -2 and -3) or the absence of light, UV radiation, ionizing radiation, and high or low osmotic pressures. In a particularly preferred embodiment, the resistance to a toxic effect is a resistance to an antibiotic such as ampicillin.

Strains generally useful in the invention can be grown on 1X Vogel's Minimal Medium (N medium) in cotton-plugged test tubes, with supplements being added depending on the phenotype of the strain, such as, for example, histidine, arginine and/or inositol. Typical strains may be obtained, for example, from the Fungal Genetics Stock Center ("FGSC") and from D.D. Perkins, Stanford University. Another *N. crassa* strain believed to be useful is M246-89601-2A (obtained from Dr. Mary Case, University of Georgia, Athens). This strain is a derivative of wild-type 74A, which contains a stable qa-2 mutation (M246), an arom-9 mutation (M6-11), and an inos (io601) mutation. The double mutant qa-2, arom-9, lacks both the biosynthetic and catabolic

dehydroquinase activities and is unable to grow on minimal medium without a supplement of aromatic amino acids, such as, for example, phenylalanine at a concentration of about 80 μg per ml.

Useful strains of *A. niger* (ATCC 46951) can be prepared by mutagenizing with UV light to form an isolate that requires ornithine or arginine for growth in a defined minimal media. This strain, which lacks ornithine carbamoyl transferase, has been called arg B (350(-)52). Media for growing *A. niger* or *A. nidulans* are described by Cove, Biochim Biophys Acta (1966) 113:51-56.

Standard procedures are generally used for the maintenance of strains and the preparation of conidia (Davis and de Serres, Methods Enzymol (1971) 17A:79-141). Mycelia are typically grown in liquid cultures for about 14 hours (25°C), as described in Lambowitz *et al.* J Cell Biol (1979) 82:17-31. Host strains can generally be grown in either Vogel's or Fries minimal medium supplemented with the appropriate nutrient(s), such as, for example, histidine; arginine; phe, tyr, and/or trp (each about 80 μg per ml); p-aminobenzoic acid (about 2 μg per ml); and inositol (about 0.2 mg per ml).

Many fungal strains with the desired characteristics are publicly available. If not readily available, however, one of ordinary skill in the art can use selection techniques well-known in the art for separating out either the desired mutants or the engineered nuclei providing the desired characteristic. Illustrative parental combinations are shown in the table below.

Table 2

First Nucleus		Second Nucleus		
First Characteristic	Second Property	Second Characteristic	First Property	Fusion Conditions
his^-	arg^+	arg^-	his^+	minimal medium (MM)
his^-	bm^r	bm^s	his^+	MM+bm
$cyclohex^s$	bm^r	bm^s	$cyclohex^r$	MM+bm +cyclohex

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caffeine[s]	arg[+]	arg[-]	caf-1	MM +caffeine
Thi-2	wt	aro-6	wt	MM +thiamine +trp+phe

As seen in the table, a variety of complementary characteristic/property combinations can be chosen to fit various fusion conditions. In general, the nutrient requirement is manifested by a mutant strain, while the ability to resist certain substances may more conveniently be conferred by modification of the nucleus with an expression system for the resistance gene. Alternatively, the nutritional requirement can be effected using recombinant techniques such as homologous recombination with a transforming vector and the resistance can be conferred by mutation under conditions where the toxic conditions are present.

Construction of Expression Vectors for Heterologous Dimer Subunits

The expression systems containing nucleotide sequences encoding a subunit of a heterologous heterodimer are constructed using well known techniques by inserting the coding sequences into host vectors and into operable linkage with control sequences which are capable of effecting their expression in the ultimate filamentous fungus host.

Intermediate hosts are sometimes used to produce intermediate vectors capable of transforming the ultimate fungal cells. The intermediate bacterial transformants can then be grown to obtain the desired quantities of DNA, which can be used to transform a desired filamentous fungus host. Examples of commonly available bacterial vectors that can serve as intermediate vectors include, for example, pBR322, pUC8 and pUC9. Additional useful intermediate vectors include pHY201, pKBY2, pTZ18R, pX182 and pCVN2.9, pN807, pN846.

Alternatively, the sequences encoding the desired subunit can be amplified using standard amplification techniques such as PCR. The coding sequences are then inserted into suitable vectors operably linked to control sequences which

affect their expression in filamentous fungi. These vectors can conveniently contain a selectable marker so that successful transformants can easily be identified. The host strain will have characteristics, however, which facilitate its fusion with a complementary host strain as described above.

Thus, to modify the nucleus of the first fungus host strain to contain an expression system for a DNA encoding a particular subunit of the desired heterologous heterodimer, the practice of the invention employs, unless otherwise indicated, molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis *et al.*, Molecular Cloning: A Laboratory Manual (1982); D.N. Gover *et al.* DNA Cloning: A Practical Approach (1985) Volumes I and II; Oligonucleotide Synthesis (M.J. Gait ed. 1984); Nuclei Acid Hybridization (Hames *et al.* eds. 1985); Transcription and Translation (Hames *et al.* eds. 1984); Animal Cell Culture (R.I. Freshney ed. 1986); Immobilized Cells and Enzymes (IRL Press 1986); B. Perbal, A Practical Guide to Molecular Cloning (1984).

In describing the invention, the following terminology will be used in accordance with the definitions set out below:

A "recombinant host" refers to cells that have been, are or will be transformed with DNA sequences prepared by recombinant techniques, and includes the cell originally transformed and cultures and progeny thereof.

A cell has been "transformed" by exogenous DNA when such exogenous DNA has been introduced into the host cell membrane. For prokaryotes such as bacteria the exogenous DNA may be maintained on an episomal element such as a plasmid. Because filamentous fungi do have nuclei (are eukaryotic), most stably transformed fungus host cells contain the exogenous DNA integrated into a chromosome, so that it is inherited by daughter cells through chromosome replication.

A "heterologous" region of a DNA construct is an identifiable segment of DNA within a large DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a gene, the gene will

usually be flanked by DNA that does not flank the genomic DNA in the genome of the source organism.

The invention involves the production of "heterologous heterodimers" in the filamentous fungi. In this context, "heterologous" means that the heterodimer is not ordinarily produced by the fungus. "Heterodimer" means that the ultimate product is made up of at least two different subunits. The dimer may be repeated in the ultimate product as is the case with immunoglobulins. Thus, heterodimers include biological materials having two or more distinct sub-units, often designated as "alpha" portions ("α") and "beta" ("β") portions. Examples include prokaryotic or eukaryotic enzymes, blood proteins, hormones, growth factors, toxins and other proteins from pathogens for vaccines, structural proteins, lymphokines, membrane surface proteins, immunoglobulin, enzyme regulators, transcription regulators, and the like.

Preferred heterodimeric proteins include α- and β-transforming growth factors, α'- and β'-antitrypsin, an immunoglobulin, insulin, hemoglobin, an α- and β-kinase, FSH, LH, hCG, and TSH. Particularly preferred heterodimeric proteins include an immunoglobulin, insulin, FSH, LH, hCG and TSH.

A "nucleotide sequence encoding" a protein is that portion of a sequence for which the transcript is translated into a polypeptide when operably linked to appropriate control sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3' (carboxy) terminus. This coding sequence can be derived from, for example, prokaryotic genes, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic DNA (such as mammalian), or may include synthetic DNA. A polyadenylation signal and transcription termination sequence will usually be located 3' to the coding sequence.

A coding sequence is "operably linked to" control sequences when the control sequences effect the expression of the coding sequence in the appropriate host cell.

An "expression system" is a DNA that contains a coding sequence operably linked to the regions of expression control necessary for expression in a host organism.

In one embodiment of the invention, host cells are converted to spheroplasts for transformation. When spheroplasts are used, a preferred method or preparing them is by enzymatic digestion of the cell walls, for example, by using a chitinase/glutamase mixture. The selection of a suitable enzyme for enzymatic digestion is within the skill of the art. Useful enzymes are those capable of digesting complex polysaccharides, and are found among those known as effective in preparing fungal spheroplasts of a wide variety of fungal species. Specific examples of suitable enzymes include Novozym **234™ (an impure** mixture of enzymes) and β-glucuronidase. Other suitable methods may be used to form spheroplasts. If suitable methods for cell wall penetration by the use of vectors are identified, however, whole cells of the fungal host may be used along with or instead of spheroplasts.

A general procedure for transforming *Neurospora* is provided below.

General Procedure for Transformation of N. crassa

Strains of *Neurospora crassa* generally used include those publicly available from the Fungal Genetics Stock Center, but independently prepares strains can also be used. Mutants may be isolated *de novo*, as illustrated by Stadler *et al.* Genetics (1966) 54:677-685 and Haas *et al.* Genetics (1952) 37:217-26. Useful strains can also be obtained from D.D. Perkins from Stanford University. Strains are typically grown on 1X Vogel's Minimal Medium ("N medium") in cotton-plugged test tubes, with appropriate supplements being added depending on the strain's phenotype.

Spheroplasts are used as subjects for transformation. To form conidial spheroplasts, the fungus is inoculated onto 25 ml of solid N medium, with appropriate supplements in four to five 125-ml Erlenmeyer flasks, which have been plugged with cotton. The cultures are grown at room temperature for 5-7 days.

The conidia are harvested by adding 10 ml of N medium to each flask, replacing the cotton plug, and swirling the flask. The solids are allowed to settle for a few minutes. The

conidial mixture is poured to an autoclaved cheesecloth bag hanging in the mouth of an Erlenmeyer flask and secured with one or more rubber bands. The filtrate is recovered, and the concentration of conidia is determined by a hemocytometer count, with chains being counted as one.

A volume of 2 X 10^9 conidia is added to 150 ml of liquid N medium containing 1.5% sucrose and appropriate supplements. The conidia are germinated in the cotton-plugged flask while shaking (150-200 rpm) for 5-6 hours at room temperature until more than 75% have germinated and the germ tubes are 1-4 conidial diameters in length. The cells are harvested by centrifuging at about 1500-2000 rpm for 10 minutes. The cell pellet is rinsed three times with water.

The pellet is then re-suspended in 10 ml of 1.0 M sorbitol, and the spheroplasts are prepared by enzymatic removal of the tough conidial cell wall with an enzyme under isotonic conditions, to prevent the "bursting" of the spheroplasts as they are formed. The protocol is adapted from the method of Vollmer and Yanofsky, Proc Natl Acad Sci USA (1986) 83:4869-73.

Specifically, in a sterile 250 ml Erlenmeyer flask, the conidial suspension is generally added to 50 mg of a solid enzyme sold by Novo Laboratories under the trade name Novozym 234. The mixture is shaken (100 rpm) at 30°C for about an hour (±10 minutes) to digest the cell wall. The spheroplast formation process is monitored by examining a small aliquot of the mixture microscopically under a cover slip. Spheroplasts can be detected because they lyse osmotically when water is applied to one end of the cover slip. The process should be monitored frequently at the later stages of spheroplast formation.

The spheroplast mixture is decanted into a sterile 15-ml conical centrifuge tube, and the spheroplasts are recovered by centrifuging at 500 rpm (10 minutes) in a swinging bucket table top centrifuge. The resulting pellet is rinsed twice with 10 of 1.0 M sorbitol and then once with the following STC solution:

91 g sorbitol;

50 mM Tris. Cl;

50 mM $CaCl_2$;

sufficient NaOH to adjust the pH to 8.0; and

sufficient water to make a volume of 500 ml.

The final spheroplast pellet is suspended in a mixture of 16.0 ml STC, 200 μl DMSO, and 4 ml of the following PTC solution:

200 g polyethylene glycol sold under the trade name "4000" by Sigma;

50 mM Tris. Cl;

50 mM $CaCl_2$;

sufficient NaOH to adjust the pH to 8.0; and

sufficient water to make a volume of 50 ml.

The resulting suspension of spheroplasts can either be used directly or stored frozen in 1.0 ml aliquots at -80°C.

In a sterile, 15-ml screw-cap tube, 2.0 μl of 50 mM Spermidine solution, 5.0 μl of the plasmid DNA to be transfected, such as that containing the expression system for a subunit of the desired heterodimer along with a selectable marker such as benomyl resistance (usually at a concentration of about 1.0 mg/ml) and 5.0 μl of a 5 mg/ml heparin solution are mixed by flicking the tube. The spermidine solution is prepared by dissolving 12.73 mg of spermidine in 1.0 ml TE and adjusting the pH to 8.0, and can be stored at -20°C. The heparin solution is prepared by dissolving 50 mg of the sodium salt of heparin in 10 ml of STC and can be stored in frozen aliquots.

The contents of the tube are briefly spun (pulsed) in a tabletop centrifuge and then placed in an ice bath. About 50-100 μl of thawed spheroplasts are added to the tube. The mixture is then incubated on ice about 30 minutes, but incubation periods of about 20 minutes on ice have been successful. About 1 ml of PTC is added and mixed well by flicking the tube. The mixture is incubated further at room temperature for about 20 minutes.

A Regeneration "Top" Agar is prepared by mixing:

20 ml 50X Vogel's Minimal Medium;

825 ml of water;

182 g sorbitol; and

28 g of agar sold under the trade name

Bacto-Difco.

The top agar is autoclaved and 100 ml of a 10X FIGS solution (containing 5 g/l fructose, 2 g/l inositol, 2 g/l glucose, and 200 sorbose) is added. 15 ml of the top agar is incubated at 50-55°C and poured into the tube containing the spheroplasts and plasmid DNA. The contents are quickly mixed by flicking and inverting the tube 2-3 times and then uniformly poured onto a layer of plating "bottom" agar.

The "bottom" agar is prepared by mixing any required supplements, in 1X N medium; autoclaving; and adding 10X FIGS and benomyl (if benomyl resistance is used as a marker) to final concentrations of 1X and 0.5 μg/ml respectively. A volume of 25 ml of "bottom" agar is poured into a petri plate and allowed to harden.

After the top agar has been poured over the bottom agar, bubbles are removed by flaming. The plates are kept in an upright position until the top agar has solidified (about 5 minutes). If the top agar tends to harden prematurely, the bottom agar plates can be prewarmed. Once the top agar has solidified, the plates are incubated in an inverted position at 30°C.

For selection of the *N. crassa* transformants, the host is thus cultured on the appropriate medium (having composition only the transformed cells can utilize or containing an antibiotic to which only transformed cells are resistant) and incubated at about 34°C. An indication of a successful transformation can be seen about 24-36 hours after plating. Stable transformants are generally scored after three days of growth. The incubation period to detect transformants will vary depending on the host strain and the phenotypic marker.

Selected transformants can be screened, expression of the desired protein subunit by standard methods, such as an appropriate ELISA, a colony blot immunoassay, restriction enzyme analysis, filter hybridization, nested deletion subcloning, and the like.

In the present invention, the above-described recombinant techniques are used to produce:

(1) a first fungus having a first characteristic that negatively affects growth under specified conditions but is correctable by a property conferred by a second nucleus; the first fungus now transformed to contain an expression system for a nucleotide sequence encoding a first heterodimer subunit; and

(2) a second fungus having a second characteristic that negatively affects growth under specified conditions but is correctable by a property conferred by the first nucleus; the second fungus now contains an expression system for a nucleotide sequence encoding the second subunit, which is different from the first subunit.

The resulting first and second strains are the parents used to form the heterokaryons of the invention.

Production of the Heterokaryon

Because the first fungus strain and the second fungus strain are chosen to be homozygous with respect to all heterokaryon compatibility alleles (with the exception of the mating allele when the tol gene is present as explained above), when the first and second fungus are cultured together under conditions wherein neither the first fungus nor the second fungus can survive alone the fungi are fused so that the heterokaryotic fungus of the invention is formed. By hyphal fusion, the different haploid nuclei of the first and second fungi come to coexist in a common cytoplasm. While not wishing to be bound by any theory, applicants believe membrane fusion results from the aggregation of intramembranous particles within each cell, making possible cell contacts between protein-free areas. Rearrangement of the lipids in the contact areas then leads to full fusion.

Because each of the two parents contains a nucleus which effects production of different subunit of the heterodimeric protein desired, the resulting heterokaryon is capable of producing the completed heterodimer comprising both subunits.

The invention heterokaryon is stable, with the two nuclei dividing at about the same rate. When heterokaryons having two (or more) nuclei are formed, it is also possible to form some mononucleated hybrid cells if the nuclei enter mitosis at approximately the same time as they fuse. This type of nuclear fusion does yield heterozygous diploid nuclei when it occurs, but it is rare, and the diploid nuclei formed are usually greatly outnumbered by the haploid nuclei.

Culture Conditions for Production of Heterodimers

The fused, heterokaryotic fungus is maintained under conditions wherein neither the first nor second fungus is viable. For example, if each of the fusing fungal strains carry an auxotrophic requirement different from the other, the only cells capable of growing in culture media where both of the nutrients are absent will be complementary heterokaryons which are also capable of expressing the subunits of the heterodimeric protein. For example, one strain may require an amino acid, such as arginine, while the other strain may require a base, such as adenine. Each strain can be independently maintained on media supplemented with the appropriate extra metabolite, but neither strain can survive alone on minimal media. The heterokaryons, however, will survive on minimal media because each nucleus complements the other's requirement.

A typical minimal medium is shown below:

MINIMAL MEDIUM

Per liter:	Dextrose	5.0 g
	Salt solution (below)	50.0 ml
	Trace elements (below)	1.0 ml
	± Agar (Difco)	12.5 g

Adjust to pH 6.5; autoclave 15 minutes.

SALT SOLUTION

Per liter:	$NaNO_3$	120.0 g
	KCl	10.4 g
	$MgSO_4$	10.4 g
	KH_2PO_4	30.4 g

TRACE ELEMENT SOLUTION

Per liter:	$(NH_4)_6Mo_7O_{24}.4H_2O$	1.1 g
	H_3BO_3	11.0 g
	$CoCl_2.6H_2O$	1.6 g
	$CuSO_4$	1.6 g
	Na_2EDTA	50.0 g
	$FeSO_4.7H_2O$	5.0 g
	$MnCl_2.4H_2O$	5.0 g
	$ZnSO_4.7H_2O$	22.0 g

Dissolve components sequentially, boil, cool, adjust pH to 6.5 with KOH.

Thus, to maintain the heterokaryotic filamentous fungus in its heterokaryotic state, external forcing is maintained. Growing the heterokaryotic fungal cells on minimal media "forces" the strains to remain together. If mating types are opposite, the presence of the tol gene can be used to maintain stable (A + a) heterokaryons.

The heterologous dimeric protein is produced by culturing the heterokaryon of the invention under conditions favorable to production of the protein. The heterodimer may be recovered from the culture and purified in accordance with standard techniques adapted, of course, as necessary to preserve the structure of the heterodimer.

Preferably, the heterokaryotic filamentous fungus carries an expression vector that allows the host being cultured to secrete the desired heterodimeric protein directly into a minimal growth medium, so that the heterodimeric protein(s) can be purified directly from cell-free medium. Intracellularly produced heterodimer can be isolated from cell lysates. Useful purification methods in accordance with known procedures are within the skill of the art, such as, for example, molecular size exclusion, ion-exchange chromatography, HPLC, and the like.

It will be understood that this description and disclosure of the invention is intended to cover all embodiments

that are within the spirit and scope of the invention. For example, it is within the knowledge of the art to insert, delete or substitute amino acids within the amino acid sequence of an open reading frame without substantially affecting the activity of the molecule, and such heterodimeric subunits with such deletions, additions or substitutions are included in the invention.

The following examples are provided by way of illustration, but are not intended to limit the invention in any way. In these examples, all media were autoclaved. Heat-labile supplements and antibiotics were added after the media had cooled. The components of N medium can be found in the review by Davis and DeSerres, Methods Enzymol. (1970) 27A:29-143. When ampicillin is added to media, a final concentration of about 50-100 μg/ml is used.

Example 1

Insertion of Heterologous DNA into pXpress

A. An insert containing the α subunit of the human LH gene is gel purified for insertion into the vector. The complete amino acid and nucleotide sequence for this subunit are reported by Boothby, M. et al. J Biol Chem (1981) 256:5121-5127 and by Fiddes, J.C. et al. J Mol Appl Genet (1981) 1:3-18.

A host vector for expression of the heterodimeric subunits, "pXpress" was prepared as described in PCT application WO93/25663 published 23 December 1993.

This vector provides a selectable marker on media containing pfpa for transformants that are homokaryotic for this vector, and also contains an Amp^r gene.

Briefly, the illustrative vector pXpress is constructed from the vector pBN3 which is described in Stuart, W.D., *et al.*, Genome (1988) 30:198-203. pBN3 contains the 2783 bp *N. crassa* genomic DNA containing the *mtr* gene shown in Figure 1, which is bracketed by a BglII site in the gene and a BamHI site contained in the vector. pBN3 was digested with BamHI and BglII and the segment containing the *mtr* gene was inserted into the BamHI site of the commercially available vector, pTZ18R,

obtained from Pharmacia™. This produced clones pN807 and pN816 wherein the EcoRI site contained in the polylinker of the pTZ18R vector is upstream of the ORF; clones in the opposite orientation were designated pN846 and pN839. pXpress is a version of pN846 wherein the 5' polylinker of pTZ18R is deleted. The pXpress vector has useful cloning sites for insertion of the desired DNA in the upstream region just downstream of the *mtr* promoter (SalI/AccI/HincII) (position 307 in Figure 1) and also in the latter third of the ORF (HincII at position 1406 and AccI at position 1920).

Five micrograms of pN846 DNA were isolated from *E. coli* NM522 by standard methods (Koo and Stuart Genome (1991) 34:644-651). The DNA was double digested with XbaI and HindIII, treated with Klenow and NTPs, cleaned with Geneclean™ (Bio 101), and ligated with 400 units of DNA T4 polymerase at room temperature overnight. The ligation mixture was used to transform *E. coli* NM522 host cells and selected for Amp^r. Transformed colonies were picked and grown in 1.5 ml liquid cultures in tubes overnight. Plasmid DNA was isolated and tested for the presence of HindIII, XbaI and PstI restriction sites. Isolates which had lost the three sites were then tested for the remaining sites expected to be in pN846. One plasmid which had lost the expected sites and retained the expected sites was designated plasmid pXpress.

pXpress is digested with SalI and then with HincII to produce a vector having part of the mtr gene sequence removed, a SalI sticky end overhang at the 5' end at bp 307 following the mtr promoter, and a HincII blunt end site at bp 1406 within the mtr gene ORF.

A sample of 0.5 μg of the αLH subunit ORF provided with suitable restriction sites is ligated into 0.5 μg of pXpress using 40 units of T4 ligase and incubating overnight at 16°C. The fragments ligate, SalI to XhoI, and blunt end HindIII to blunt end HincII (thus losing all four restriction sites).

The ligated fragments are transformed into competent *E. coli* cells DH-5alpha and transformants selected for resistance to ampicillin. Resistant colonies are grown in liquid cultures and a standard preparation is performed to

isolate plasmid DNA. The plasmid is digested with the restriction enzyme EcoRV to test the size of the plasmid, i.e., to confirm the presence of the insert. Plasmids testing positive are then digested with BamHI to test the orientation of the insert into the plasmid. Positives are renamed for the desired subunit, pLHα.

B. In a manner exactly analogous to that of paragraph A of this example, the gene encoding LHβ subunit is inserted into pXpress to obtain pLHβ. The complete sequence of this subunit is described by Boorstein, W.R. et al. Nature (1982) 300:419-422.

Example 2

Transformation of Neurospora Spheroplasts and Expression of LH Subunit

A. *Neurospora* spheroplasts of strain Y152m14 (which requires histidine) are transformed with pLHα by standard methods. (Koo and Stuart 1991, supra.) The plasmid is linearized by cutting with SacI. A sample of 5 μg of the linearized plasmid is used to transform 1 x 10^8 spheroplasts. The mixture is taken up in 15 ml of minimal top agar supplemented with histidine and spread onto a bottom plate containing 0.05 mg/ml p-fluorophenylalanine ("pfpa"). Plates are screened three days later. Colonies are picked and grown on solid Vogel's 1X media containing 0.05 mg/ml pfpa and histidine supplement in tube slants.

Colonies are transferred to liquid cultures of 1X Vogel's with 2% sucrose and histidine in double distilled water. The culture is collected and assayed for the presence of LHα subunit.

B. *N. crassa* strain M246-89601-2A is a double mutant (qa-2, arom-9) that lacks both the biosynthetic and catabolic dehydroquinase activities. This strain is unable to grow on minimal medium without a supplement of at least one aromatic amino acid such as phenylalanine. This strain is transformed with pLHβ using the procedure of paragraph A of this example except that the media are supplemented with phenylalanine rather

than histidine. After transfer to liquid culture, as in paragraph A, the cultures are assayed for LHβ subunit.

Example 3

Heterokaryon Formation

Both the first and second transformed fungus strains of Example 2 are cultured on a minimal medium lacking histidine and phenylalanine. This medium "forces" the two strains to form heterokaryotic cells having both types of nuclei inside a single septal wall.

The fused, heterokaryotic host is maintained on minimal medium under conditions that favor expression of the α and β subunits. The correctly assembled heterodimeric LH is produced, recovered from the culture and, if desired, purified by conventional techniques.

From the foregoing, it will be appreciated that, although certain embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not to be limited except as by the appended claims.

Claims

1. A heterokaryon filamentous fungus which fungus contains a first nucleus and a second nucleus, wherein said first nucleus has been modified to contain an expression system for a first nucleotide sequence encoding a first subunit of a heterologous heterodimer and said second nucleus has been modified to contain an expression system for a second nucleotide sequence encoding a second subunit of said heterologous heterodimer.

2. The fungus of claim 1, wherein the first nucleus confers a first characteristic negatively affecting growth under specified conditions that is correctable by a first property conferred by the second nucleus and the second nucleus confers a second characteristic negatively affecting growth under said specified conditions that is correctable by a second property conferred by the first nucleus under said specified conditions.

3. The fungus of claim 2, wherein the first characteristic is a requirement for a first nutrient; the first property is lack of said requirement; and the specified conditions comprise culture in a medium that lacks said first nutrient.

4. The fungus of claim 3, wherein the second characteristic is a requirement for a second nutrient; the second property is lack of said requirement; and the specified conditions comprise culture in a medium that lacks said first and second nutrients.

5. The fungus of claim 2, wherein the first characteristic is inability to grow in the presence of a first toxic substance; the first property confers resistance to said first toxic substance; and the specified conditions comprise culture in a medium that contains said first toxic substance.

6. The fungus of claim 5, wherein the second characteristic is inability to grow in the presence of a second toxic substance; the second property confers resistance to said second toxic substance; and the specified conditions comprise culture in a medium that contains said first and second toxic substances.

7. The fungus of claim 3, wherein the second characteristic is inability to grow in the presence of a toxic substance; the second property confers resistance to said toxic substance; and wherein said specified conditions comprise culture in a medium that lacks said first nutrient and contains said toxic substance.

8. A method to produce a heterodimer heterologous to a filamentous fungus, which method comprises culturing the fungus of any one of claims 1 to 7, under conditions wherein said first and second subunits are produced to form said heterodimer; and

recovering the heterodimer from the culture.

9. A method to prepare the heterokaryon fungus of claim 1, which method comprises:

culturing a first fungus containing said first nucleus, wherein said first nucleus confers a first characteristic negatively affecting growth under specified

conditions that is correctable by a first property conferred by the second nucleus,

along with a second fungus containing said second nucleus wherein said second nucleus confers a second characteristic negatively affecting growth under said specified conditions that is correctable by a second property conferred by the first nucleus;

said culturing being conducted under said specified conditions.

10. The method of claim 9, wherein the first characteristic is a requirement for a first nutrient; the first property is lack of said requirement; and the specified conditions comprise culture in a medium that lacks said nutrient.

11. The method of claim 10, wherein the second characteristic is a requirement for a second nutrient; the second property is lack of said requirement; and the specified conditions comprise culture in a medium that lacks said first and second nutrients.

12. The method of claim 9, wherein the first characteristic is inability to grow in the presence of a first toxic substance; the first property confers resistance to said first toxic substance; and the specified conditions comprise culture in a medium that contains said toxic substance.

13. The method of claim 12, wherein the second characteristic is inability to grow in the presence of a second toxic substance; the second property confers resistance to said second toxic substance; and the specified

conditions comprise culture in a medium that contains said first and second toxic substances.

14. The method of claim 10, wherein the second characteristic is inability to grow in the presence of a toxic substance; the second property confers resistance to said toxic substance; and wherein said specified conditions comprise culture in a medium that lacks said first nutrient and contains said toxic substance.

15. The method of any one of claims 9 to 14, further comprising transforming a filamentous fungus with an expression vector encoding a first subunit of a heterologous heterodimer to produce said first fungus.

16. The method of any one of claims 9 to 15, further comprising transforming a filamentous fungus with an expression vector encoding a second subunit of a heterologous heterodimer to produce said second fungus.

17. The fungus of any one of claims 1 to 7, wherein said heterodimer is selected from the group consisting of follicle stimulating hormone (FSH), leuteinizing hormone (LH), human chorionic gonadotropin (hCG), thyroid stimulating hormone (TSH), insulin, and an immunoglobulin.

AGATCCGCCT CGCCCCAAGC GCATCCCAAC GCGGCGTGCT TATATGTCGC TCTTCCCTCT 60

CACGTACCTC GCAAGTACCT GTCTCATCTG GCACCCGCCT TCTCCATCCC TCTTCTTCAG 120

TACTTAATCT GCCCCGGTCC CCCGCAGTTC ATCCTGTCTC TCAGACCTTG GATCGTCTGT 180

TGGTCTTTTC GCTTATTGTC CGTACCCCCA TCCATATTTA TTCCTGCCTG GGCCCCCAGA 240

CAGCATTTTC TCTTTCCCTC CCTCTTTAGC CATCCAAACA GCTTGAGAAG CGAAAATCAA 300

GCCACTGTCG ACAAAGGCCT TCAACAAGGC CTCTTTACCA CCCAACATGG ACTCGCAATA 360

CGAGACAAAA AAGAATGACC CAAACGCCAT CATGCCGTAC CCAGAGTCAA ACGATGAGCA 420

TGTTGGCGAG GTCCGCGGCT TGGGCGGCGG CATCATGGAC AAGGAGCCTG AGGCCCAGGA 480

GGGCCATGCC AAGTTCCACC GTCTCGGCTG GAAACGTCTG ACGGTCGTCC TCATCGTCGA 540

GGCCATTGCC CTCGGCTCTC TCTCGCTTCC CGGCGCCTTC GCTACCCTTG GCATGGTGCC 600

TGGTGTTATT CTCTCTGTCG GCATGGGACT CATCTGCATC TACACGGCTC ACGTTATCGG 660

ACAAACCAAG CTCAAGCACC CTGAAATCGC CCACTATGCC GACGTTGGTC GTGTCATGTT 720

TGGAAGATGG GGATATGAAA TCATCAGCTT CATGTTTGTT CTGCAACTGA TCTTCATCGT 780

CGGCTCCCAC GTCCTCACTG GCACCATCAT GTGGGGCACC ATCACGGATA ACGGCAACGG 840

TACCTGCTCT CTCGTCTTCG GCATTGTCTC CGCCATCATT CTCTTCCTCC TTGCCATTCC 900

TCCCAGTTTC GCCGAGGTTG CCATCCTTGG ATACATCGAT TTCGTCTCCA TCTGCGCCGC 960

CATCCTCATC ACCATGATTG CTACTGGCAT TCGCTCGAGC CACCAGGAGG GTGGTCTCGC 1020

TGCTGTTCCC TGGTCTTGCT GGCCCAAGGA GGACCTTAGC CTTGCTGAGG GCTTCATTGC 1080

TGTCAGCAAC ATCGTTTTCG CCTACAGCTT CGCCATGTGC CAGTTCAGCT TTATGGATGA 1140

GATGCACACC CCCTCCGACT ACAAGAAGTC CATCGTTGCT CTCGGCTTGA TTGAAATCTT 1200

CATCTACACC GTTACTGGTG GCGTCGTTTA CGCTTTCGTC GGCCCCGAGG TCCAGTCTCC 1260

TGCCTTGCTC TCTGCTGGCC CTCTTCTCGC CAAGGTTGCT TTCGGCATTG CCCTCCCCGT 1320

FIGURE 1

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CATCTTCATC TCTGGCAGTA TCAACACTGT TGTCGTCAGC AGGTATCTGA TTGAGCGCAT    1380

CTGGCCCAAC AACGTCATTC GCTATGTCAA CACCCCAGCG GGTTGGATGG TTTGGCTTGG    1440

TTTTGACTTT GGCATTACCC TCATTGCCTG GGTTATTGCT GAGGCCATCC CTTTCTTCTC    1500

TGATCTGTTG GCCATCTGCT CGGCTCTCTT CATTTCCGGT TTTAGCTTCT ATTTCCCTGC    1560

CTTGATGTAT TTCAAGATCA CCAGGAACGA TGCCAAGAGC CAGGGCAAGA AGTACTTCTT    1620

GGATGCCCTC AACATGCTCT GCTTCGTCAT CGGCATGGGC ATTCTTGGTA TTGGTACCTA    1680

CGCCGCTATT CAGGACATTG TAAGTTTGGC CCGCTTTTCT GTTTACTCTT TGCACACAAA    1740

TGCTAACTTG CTTCTCAGAT GGACCGTTAC GACCATGGCA AGGTTTCGAA GCCTTATAGC    1800

TGTGCGCCCT TGGCTTAACA GGCCCAACGC ACGCTTATGA TCCTGTTGTT TTTTTTTGGA    1860

TGATTTAATT AAAGTTGCGC AGTGATTGAC GTCTGTCTTC ACCCGCGATT GCCCCTTTTG    1920

TATACCCCCT CAGACTTGCC GGCCTGGGGA AATGTTTTGA GTATTTCTAT TTTCGGAGTT    1980

TCAGGATTTG GCACAAAGCA AACCAGCGCG GAGTTGAAAC CGTGGTGTGG TCGCGGTGCG    2040

CTGCTGCATT GGTAGTGCTT GTTCCAGGTT TTTGTTTGGT GGTTTGGATG CGTGCACCAC    2100

TTTTTTTTTT AACGTTTTAT TGCATGCATG TATTATATGG GAAAGTCATG GGACATGGCA    2160

ACTATACGAA CCGACGCAAA GATAGGATGG GATGGATGAT GGATGGACGT ACGATCCAAC    2220

GCGCTGGGGA CTGGACTGAA CGGAATTAGG ACGGACGGGA CAGGTAACCT AGGTACCTAA    2280

TGACCGGAAT ATGTTTACAA ATCATTGTTT AGTGCGGGTG ACCGGCAATA GAGACGATGG    2340

GCACAGGAAT ATCGATAGAT GCTACCTATA CTCTAAAGAA CTCTATAGGT ATAATATTCG    2400

CTGAACATAC CTTGCCCAAA AAACAAGAGA ACACCCATGG TTATGAAATC ATCCTGTTGT    2460

TGTGCCATAA TTTCCATCCT GACTCCCATG CCTTCCTGCT TTTTTTTTTT TTTTTTCTTG    2520

GCCACGCGCC TCCGATATCT CGAGTTTTTG AAGGATTCTC GTGTTGGGTG GAGCTTTTCT    2580

CAACAATCCT GGGGCTTCGA ATCCCTCCAC CAGACCTCAC CCCAGCAGTC AGAGTTTAGC    2640
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FIGURE 1(cont.)

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CCGCCAGCCA GCCCGTCAGC CAAGTGAGTT CTAAGATTAA TCTCGACTCC TTGACAAGGC    2700

TTTGCGTGGC CACGTCTCCT CTCAACACGC AAAACTTTTG TCATTGTTAC TACACTACAG    2760

GTTACCGTGT CGATGTTCCA GGC                                          2783
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FIGURE 1(cont.)

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ATG GAC TCG CAA TAC GAG ACA AAA AAG AAT GAC CCA AAC GCC ATC ATG          48
Met Asp Ser Gln Tyr Glu Thr Lys Lys Asn Asp Pro Asn Ala Ile Met
  1               5                  10                  15

CCG TAC CCA GAG TCA AAC GAT GAG CAT GTT GGC GAG GTC CGC GGC TTG          96
Pro Tyr Pro Glu Ser Asn Asp Glu His Val Gly Glu Val Arg Gly Leu
            20                  25                  30

GGC GGC GGC ATC ATG GAC AAG GAG CCT GAG GCC CAG GAG GGC CAT GCC         144
Gly Gly Gly Ile Met Asp Lys Glu Pro Glu Ala Gln Glu Gly His Ala
        35                  40                  45

AAG TTC CAC CGT CTC GGC TGG AAA CGT CTG ACG GTC GTC CTC ATC GTC         192
Lys Phe His Arg Leu Gly Trp Lys Arg Leu Thr Val Val Leu Ile Val
    50                  55                  60

GAG GCC ATT GCC CTC GGC TCT CTC TCG CTT CCC GGC GCC TTC GCT ACC         240
Glu Ala Ile Ala Leu Gly Ser Leu Ser Leu Pro Gly Ala Phe Ala Thr
65                  70                  75                  80

CTT GGC ATG GTG CCT GGT GTT ATT CTC TCT GTC GGC ATG GGA CTC ATC         288
Leu Gly Met Val Pro Gly Val Ile Leu Ser Val Gly Met Gly Leu Ile
                85                  90                  95

TGC ATC TAC ACC GCT CAC GTT ATC GGA CAA ACC AAG CTC AAG CAC CCT         336
Cys Ile Tyr Thr Ala His Val Ile Gly Gln Thr Lys Leu Lys His Pro
            100                 105                 110

GAA ATC GCC CAC TAT GCC GAC CTT GGT CGT GTC ATG TTT GGA AGA TGG         384
Glu Ile Ala His Tyr Ala Asp Leu Gly Arg Val Met Phe Gly Arg Trp
        115                 120                 125

GGA TAT GAA ATC ATC AGC TTC ATG TTT GTT CTG CAA CTG ATC TTC ATC         432
Gly Tyr Glu Ile Ile Ser Phe Met Phe Val Leu Gln Leu Ile Phe Ile
    130                 135                 140

GTC GGC TCC CAC GTC CTC ACT GGC ACC ATC ATG TGG GGC ACC ATC ACG         480
Val Gly Ser His Val Leu Thr Gly Thr Ile Met Trp Gly Thr Ile Thr
145                 150                 155                 160

GAT AAC GGC AAC GGT ACC TGC TCT CTC GTC TTC GGC ATT GTC TCC GCC         528
Asp Asn Gly Asn Gly Thr Cys Ser Leu Val Phe Gly Ile Val Ser Ala
                165                 170                 175

ATC ATT CTC TTC CTC CTT GCC ATT CCT CCC AGT TTC GCC GAG GTT GCC         576
Ile Ile Leu Phe Leu Leu Ala Ile Pro Pro Ser Phe Ala Glu Val Ala
            180                 185                 190

ATC CTT GGA TAC ATC GAT TTC GTC TCC ATC TGC GCC GCC ATC CTC ATC         624
Ile Leu Gly Tyr Ile Asp Phe Val Ser Ile Cys Ala Ala Ile Leu Ile
        195                 200                 205

ACC ATG ATT GCT ACT GGC ATT CGC TCG AGC CAC CAG GAG GGT GGT CTC         672
Thr Met Ile Ala Thr Gly Ile Arg Ser Ser His Gln Glu Gly Gly Leu
    210                 215                 220

GCT GCT GTT CCC TGG TCT TGC TGG CCC AAG GAG GAC CTT AGC CTT GCT         720
Ala Ala Val Pro Trp Ser Cys Trp Pro Lys Glu Asp Leu Ser Leu Ala
225                 230                 235                 240

GAG GGC TTC ATT GCT GTC AGC AAC ATC GTT TTC GCC TAC AGC TTC GCC         768
Glu Gly Phe Ile Ala Val Ser Asn Ile Val Phe Ala Tyr Ser Phe Ala
                245                 250                 255
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FIGURE 2

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ATG TGC CAG TTC AGC TTT ATG GAT GAG ATG CAC ACC CCC TCC GAC TAC          816
Met Cys Gln Phe Ser Phe Met Asp Glu Met His Thr Pro Ser Asp Tyr
            260                 265                 270

AAG AAG TCC ATC GTT GCT CTC GGC TTG ATT GAA ATC TTC ATC TAC ACC          864
Lys Lys Ser Ile Val Ala Leu Gly Leu Ile Glu Ile Phe Ile Tyr Thr
        275                 280                 285

GTT ACT GGT GGC GTC GTT TAC GCT TTC GTC GGC CCC GAG GTC CAG TCT          912
Val Thr Gly Gly Val Val Tyr Ala Phe Val Gly Pro Glu Val Gln Ser
    290                 295                 300

CCT GCC TTG CTC TCT GCT GGC CCT CTT CTC GCC AAG GTT GCT TTC GGC          960
Pro Ala Leu Leu Ser Ala Gly Pro Leu Leu Ala Lys Val Ala Phe Gly
305                 310                 315                 320

ATT GCC CTC CCC GTC ATC TTC ATC TCT GGC AGT ATC AAC ACT GTT GTC         1008
Ile Ala Leu Pro Val Ile Phe Ile Ser Gly Ser Ile Asn Thr Val Val
                325                 330                 335

GTC AGC AGG TAT CTG ATT GAG CGC ATC TGG CCC AAC AAC GTC ATT CGC         1056
Val Ser Arg Tyr Leu Ile Glu Arg Ile Trp Pro Asn Asn Val Ile Arg
            340                 345                 350

TAT GTC AAC ACC CCA GCG GGT TGG ATG GTT TGG CTT GGT TTT GAC TTT         1104
Tyr Val Asn Thr Pro Ala Gly Trp Met Val Trp Leu Gly Phe Asp Phe
        355                 360                 365

GGC ATT ACC CTC ATT GCC TGG GTT ATT GCT GAG GCC ATC CCT TTC TTC         1152
Gly Ile Thr Leu Ile Ala Trp Val Ile Ala Glu Ala Ile Pro Phe Phe
    370                 375                 380

TCT GAT CTG TTG GCC ATC TGC TCG GCT CTC TTC ATT TCC GGT TTT AGC         1200
Ser Asp Leu Leu Ala Ile Cys Ser Ala Leu Phe Ile Ser Gly Phe Ser
385                 390                 395                 400

TTC TAT TTC CCT GCC TTG ATG TAT TTC AAG ATC ACC AGG AAC GAT GCC         1248
Phe Tyr Phe Pro Ala Leu Met Tyr Phe Lys Ile Thr Arg Asn Asp Ala
                405                 410                 415

AAG AGC CAG GGC AAG AAG TAC TTC TTG GAT GCC CTC AAC ATG CTC TGC         1296
Lys Ser Gln Gly Lys Lys Tyr Phe Leu Asp Ala Leu Asn Met Leu Cys
            420                 425                 430

TTC GTC ATC GGC ATG GGC ATT CTT GGT ATT GGT ACC TAC GCC GCT ATT         1344
Phe Val Ile Gly Met Gly Ile Leu Gly Ile Gly Thr Tyr Ala Ala Ile
        435                 440                 445

CAG GAC ATT GTAAGTTTGG CCCGCTTTTC TGTTTACTCT TTGCACACAA                 1393
Gln Asp Ile
    450

ATGCTAACTT GCTTCTCAG ATG GAC CGT TAC GAC CAT GGC AAG GTT TCG AAG        1445
                     Met Asp Arg Tyr Asp His Gly Lys Val Ser Lys
                       1               5                  10

CCT TAT AGC TGT GCG CCC TTG GCT TAA                                    1472
Pro Tyr Ser Cys Ala Pro Leu Ala
            15
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FIGURE 2(cont.)