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(54) METHODES D'IDENTIFICATION D'INHIBITEURS DE L'ASPARAGINE SYNTHASE, DE LA 5-AMINOLEVULINATE SYNTHASE, DE L'HISTIDINOL PHOSPHATASE, DE LA 3-ISOPROPYLMALATE SYNTHASE ET DE LA THREONINE SYNTHASE EN TANT QU'ANTIBIOTIQUES

(54) METHODS FOR THE IDENTIFICATION OF INHIBITORS OF ASPARAGINE SYNTHASE, 5-AMINOLEVULINATE SYNTHASE, HISTIDINOL-PHOSPHATASE, 3-ISOPROPYLMALATE AND THREONINE SYNTHASE AS ANTIBIOTICS

(57)

The present inventors have discovered that Asparagine Synthase, 5- Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase are essential for fungal pathogenicity. Specifically, the inhibition of Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase gene expression in fungi results in no signs of successful infection or lesion. Thus, Asparagine Synthase, 5-Aminolevulinate synthase, histidinol- phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase can be used as targets for the identification of antibiotics, preferably antifungals. Accordingly, the present inventio provides methods for the identification of compounds that inhibit Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-hosphatase, 3-Isopropylmalate dehydratase or Threonine synthase expression or activity. The methods of the invention are useful for the identification of

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(54) Titre : METHODES D'IDENTIFICATION D'INHIBITEURS DE L'ASPARAGINE SYNTHASE, DE LA 5-AMINOLEVULINATE SYNTHASE, DE L'HISTIDINOL PHOSPHATASE, DE LA 3-ISOPROPYLMALATE SYNTHASE ET DE LA THREONINE SYNTHASE EN TANT QU'ANTIBIOTIQUES

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(57) Abrégé/Abstract:
The present inventors have discovered that Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase are essential for fungal pathogenicity. Specifically, the inhibition of Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase gene expression in fungi results in no signs of successful infection or lesion. Thus, Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase can be used as targets for the identification of antibiotics, preferably antifungals. Accordingly, the present inventio provides methods for the identification of compounds that inhibit Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-hosphatase, 3-Isopropylmalate dehydratase or Threonine synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably antifungals.

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(57) Abstract: The present inventors have discovered that Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase are essential for fungal pathogenicity. Specifically, the inhibition of Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase gene expression in fungi results in no signs of successful infection or lesion. Thus, Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase can be used as targets for the identification of antibiotics, preferably antifungals. Accordingly, the present inventio provides methods for the identification of compounds that inhibit Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-hosphatase, 3-Isopropylmalate dehydratase or Threonine synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably antifungals.

METHODS FOR THE IDENTIFICATION OF INHIBITORS OF ASPARAGINE SYNTHASE, 5-AMINOLEVULINATE SYNTHASE, HISTIDINOL-PHOSPHATASE, 3-ISOPROPYLMALATE AND THREONINE SYNTHASE AS ANTIBIOTICS

FIELD OF THE INVENTION

The invention relates generally to methods for the identification of antibiotics, preferably antifungals that affect the biosynthesis of L-asparagine, heme, L-histidine, L-leucine or L-threonine.

BACKGROUND OF THE INVENTION

Filamentous fungi are the causal agents responsible for many serious pathogenic infections of plants and animals. Since fungi are eukaryotes, and thus more similar to their host organisms than, for example bacteria, the treatment of infections by fungi poses special risks and challenges not encountered with other types of infections. One such fungus is *Magnaporthe grisea*, the fungus that causes rice blast disease. It is an organism

that poses a significant threat to food supplies worldwide. Other examples of plant pathogens of economic importance include the pathogens in the genera *Agaricus, Alternaria, Anisogramma, Anthracoidea, Antrodia, Apiognomonia, Apiosporina, Armillaria, Ascochyta, Aspergillus, Bipolaris, Bjerkandera, Botryosphaeria, Botrytis, Ceratobasidium, Ceratocystis, Cercospora, Cercosporidium, Cerotelium, Cerrena, Chondrostereum, Chryphonectria, Chrysomyxa, Cladosporium, Claviceps, Cochliobolus, Coleosporium, Colletotrichium, Colletotrichum, Corticium, Corynespora, Cronartium, Cryphonectria, Cryptosphaeria, Cyathus, Cymadothea, Cytospora, Daedaleopsis, Diaporthe, Didymella, Diplocarpon, Diplodia, Discohainesia, Discula, Dothistroma, Drechslera, Echinodontium, Elsinoe, Endocronartium, Endothia, Entyloma, Epichloe, Erysiphe, Exobasidium, Exserohilum, Fomes, Fomitopsis, Fusarium, Gaeumannomyces, Ganoderma, Gibberella, Gloeocercospora, Gloeophyllum, Gloeoporus, Glomerella, Gnomoniella, Guignardia, Gymnosporangium, Helminthosporium, Herpotrichia, Heterobasidion, Hirschioporus, Hypodermella, Inonotus, Irpex, Kabatiella, Kabatina, Laetiporus, Laetisaria, Lasiodiplodia, Laxitextum, Leptographium, Leptosphaeria, Leptosphaerulina, Leucytospora, Linospora, Lophodermella, Lophodermium, Macrophomina, Magnaporthe, Marssonina, Melampsora, Melampsorella, Meria, Microdochium, Microsphaera, Monilinia, Monochaetia, Morchella, Mycosphaerella, Myrothecium, Nectria, Nigrospora, Ophiosphaerella, Ophiostoma, Penicillium, Perenniporia, Peridermium, Pestalotia, Phaeocryptopus, Phaeolus, Phakopsora, Phellinus, Phialophora, Phoma, Phomopsis, Phragmidium, Phyllachora, Phyllactinia, Phyllosticta, Phymatotrichopsis, Pleospora, Podosphaera, Pseudopeziza, Pseudoseptoria, Puccinia, Pucciniastrum, Pyricularia, Rhabdocline, Rhizoctonia, Rhizopus, Rhizosphaera, Rhynchosporium, Rhytisma, Schizophyllum, Schizopora, Scirrhia, Sclerotinia, Sclerotium, Scytinostroma, Septoria, Setosphaera, Sirococcus, Spaerotheca, Sphaeropsis, Sphaerotheca, Sporisorium, Stagonospora, Stemphylium, Stenocarpella, Stereum, Taphrina, Thielaviopsis, Tilletia, Trametes, Tranzschelia, Trichoderma, Tubakia, Typhula, Uncinula, Urocystis, Uromyces, Ustilago, Valsa, Venturia, Verticillium, Xylaria*, and others. Related organisms in the classification, oomycetes, that include the genera *Albugo, Aphanomyces, Bremia, Peronospora, Phytophthora, Plasmodiophora, Plasmopara, Pseudoperonospora, Pythium,*

Sclerophthora, and others are also significant plant pathogens and are sometimes classified along with the true fungi. Human diseases that are caused by filamentous fungi include life-threatening lung and disseminated diseases, often a result of infections by *Aspergillus fumigatus*. Other fungal diseases in animals are caused by fungi in the genera, *Fusarium*, *Blastomyces*, *Microsporum*, *Trichophyton*, *Epidermophyton*, *Candida*, *Histoplamsa*, *Pneumocystis*, *Cryptococcus*, other *Aspergilli*, and others. The control of fungal diseases in plants and animals is usually mediated by chemicals that inhibit the growth, proliferation, and/or pathogenicity of the fungal organisms. To date, there are less than twenty known modes-of-action for plant protection fungicides and human antifungal compounds.

A pathogenic organism has been defined as an organism that causes, or is capable of causing disease. Pathogenic organisms propagate on or in tissues and may obtain nutrients and other essential materials from their hosts. A substantial amount of work concerning filamentous fungal pathogens has been performed with the human pathogen, *Aspergillus fumigatus*. Shibuya *et al.* (Shibuya, K., M. Takaoka, *et al.* (1999) Microb Pathog *27*: 123 - 31 (PMID: 10455003)) have shown that the deletion of either of two suspected pathogenicity related genes encoding an alkaline protease or a hydrophobin (rodlet) respectively, did not reduce mortality of mice infected with these mutant strains. Smith *et al.* (Smith, J. M., C. M. Tang, *et al.* (1994) Infect Immun *62*: 5247 - 54 (PMID: 7960101)) showed similar results with alkaline protease and the ribotoxin restrictocin; *Aspergillus fumigatus* strains mutated for either of these genes were fully pathogenic to mice. Reichard *et al.* (Reichard, U., M. Monod, *et al.* (1997) J Med Vet Mycol 35: 189 - 96 (PMID: 9229335)) showed that deletion of the suspected pathogenicity gene encoding aspergillopepsin (PEP) in *Aspergillus fumigatus* had no effect on mortality in a guinea pig model system, and Aufauvre-Brown *et al* (Aufauvre-Brown, A., E. Mellado, *et al.* (1997) Fungal Genet Biol *21*: 141 – 52 (PMID: 9073488)) showed no effects of a chitin synthase mutation on pathogenicity. However, not all experiments produced negative results. Ergosterol is an important membrane component found in fungal organisms. Pathogenic fungi that lack key enzymes in this biochemical pathway might be expected to be non-pathogenic since neither the plant nor animal hosts contain this particular sterol. Many antifungal compounds that affect this biochemical pathway have been

described (Onishi, J. C. and A. A. Patchett (1990a, b, c, d, and e) United States Patents 4,920,109; 4,920,111; 4,920,112; 4,920,113; and 4,921,844, Merck & Co. Inc. (Rahway NJ)) and (Hewitt, H. G. (1998) Fungicides in Crop Protection Cambridge, University Press). D'Enfert *et al.* (D'Enfert, C., M. Diaquin, *et al.* (1996) Infect Immun 64: 4401 - 5 (PMID: 8926121)) showed that an *Aspergillus fumigatus* strain mutated in an orotidine 5'-phosphate decarboxylase gene was entirely non-pathogenic in mice, and Brown *et al.* (Brown, J. S., A. Aufauvre-Brown, *et al.* (2000) Mol Microbiol *36*: 1371-80 (PMID: 10931287)) observed a non-pathogenic result when genes involved in the synthesis of para-aminobenzoic acid were mutated. Some specific target genes have been described as having utility for the screening of inhibitors of plant pathogenic fungi. Bacot *et al.* (Bacot, K. O., D. B. Jordan, *et al.* (2000) United States Patent 6,074,830, E. I. du Pont de Nemours & Company (Wilmington DE)) describe the use of 3,4-dihydroxy-2-butanone 4-phosphate synthase, and Davis *et al.* (Davis, G. E., G. D. Gustafson, *et al.* (1999) United States Patent 5,976,848, Dow AgroSciences LLC (Indianapolis IN)) describe the use of dihydroorotate dehydrogenase for potential screening purposes.

There are also a number of papers that report less clear results, showing neither full pathogenicity nor non-pathogenicity of mutants. Hensel *et al.* (Hensel, M., H. N. Arst, Jr., *et al.* (1998) Mol Gen Genet 258: 553 - 7 (PMID: 9669338)) showed only moderate effects of the deletion of the *areA* transcriptional activator on the pathogenicity of *Aspergillus fumigatus*.

Therefore, it is not currently possible to determine which specific growth materials may be readily obtained by a pathogen from its host, and which materials may not. The present inventors have found that *Magnaporthe grisea* that cannot synthesize their own L-asparagine are non-pathogenic on their host organism. Previous studies of the *Saccharomyces cerevisiae* Asparagine Synthase genes, ASN1 and ASN2, indicated that disruption of ASN1 or ASN2 alone has no effect on growth (Dang *et al.* (1996) Mol Microbiol *22*: 681 - 92 (PMID: 8951815)), teaching against our finding. To date there do not appear to be any publications demonstrating an anti-pathogenic effect of the knock-out, over-expression, antisense expression, or inhibition of a gene or gene products involved in L-asparagine biosynthesis in filamentous fungi. Thus, it has not been shown that the *de novo* biosynthesis of L-asparagine is essential for fungal pathogenicity. Thus,

it would be desirable to determine the utility of the enzymes involved in L-asparagine biosynthesis for evaluating antibiotic compounds, especially fungicides. If a fungal biochemical pathway or specific gene product in that pathway is shown to be required for fungal pathogenicity, various formats of *in vitro* and *in vivo* screening assays may be put in place to discover classes of chemical compounds that react with the validated target gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

The present inventors have found that *Magnaporthe grisea* that cannot synthesize their own heme are non-pathogenic on their host organism. In addition to being a key component of respiratory cytochromes and hemoglobin, heme is the prosthetic group for many enzymes involved in the detoxification of oxygen radicals and in the metabolism of fatty acids and sterols. In yeast, *Saccharomyces cerevisiae*, mutants deficient in heme biosynthesis have been isolated and genetically studied in detail (Gollub *et al.* (1977) J Biol Chem *252*: 2846 – 54 (PMID: 323256)). The 5-aminolevulinate synthase gene has been cloned from *Aspergillus oryzae* and shown to be used as a selectable marker for the transformation of *A. oryzae* (Elrod *et al.* (2000) Curr Genet *38*: 291 - 8 (PMID: 11191214)). In humans, two 5-aminolevulinate synthase genes have been identified. Mutations in one of them, encoding an erythroid isoform, result in X-linked sideroblastic anemia (Cox *et al.* (1994) N Engl J Med *330*: 675 - 9 (PMID: 8107717)). 5-aminolevulinate synthase has been proposed as a new antimalarial target (Padmanaban and Rangarajan (2000) Biochem Biophys Res Commun *268*: 665 - 8 (PMID: 10679261)).

To date there do not appear to be any publications demonstrating an anti-pathogenic effect of the knock-out, over-expression, antisense expression, or inhibition of the genes or gene products involved in heme biosynthesis in filamentous fungi. Thus, it has not been shown that the *de novo* biosynthesis of heme is essential for fungal pathogenicity. And, thus, it would be desirable to determine the utility of the enzymes involved in heme biosynthesis for evaluating antibiotic compounds, especially fungicides. If a fungal biochemical pathway or specific gene product in that pathway is shown to be required for fungal pathogenicity, various formats of *in vitro* and *in vivo* screening assays may be put in place to discover classes of chemical compounds that

react with the validated target gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

The present inventors have found that *Magnaporthe grisea* that cannot synthesize their own L-histidine have reduced pathogenicity on their host organism. The *M. grisea* HISP1 enzyme has greatest similarity to *Schizosaccharomyces pombe* His9, as well as some similarity to *Saccharomyces cerevisiae* His2p and His9. These genes encode a distantly related family of Histidinol Phosphate Phosphatases (HolPase), which catalyzes the dephosphorylation of Histidinol Phosphate to Histidinol. This family includes the HolPase encoded by the HisJ (or ytvP) gene found in *Bacillus subtilis*. Knock-out of HisJ has yielded an auxotrophic mutant, unable to grow without Histidine supplementation (le Coq *et al.* (1999) J Bacteriol *181*: 3277 – 3280 (PMID: 10322033)). No references were found where SpHis2, ScHis2p or ScHis9 are known targets for anti-fungal/ fungicide development. However, *S. cerevisiae* mutants containing knock-outs in the His1-His7 genes have been shown to be unable to grow in elevated levels of Cu, Co, or Ni at near-neutral pH (Pearce and Sherman (1999) J Bacteriol *181*: 4774 - 4779 (PMID: 10438744)).

To date there do not appear to be any publications demonstrating an anti-pathogenic effect of the knock-out, over-expression, antisense expression, or inhibition of the genes or gene products involved in L-histidine biosynthesis in filamentous fungi. Thus, it has not been shown that the *de novo* biosynthesis of L-histidine is essential for fungal pathogenicity. And, thus, it would be desirable to determine the utility of the enzymes involved in L-histidine biosynthesis for evaluating antibiotic compounds, especially fungicides. If a fungal biochemical pathway or specific gene product in that pathway is shown to be required for fungal pathogenicity, various formats of *in vitro* and *in vivo* screening assays may be put in place to discover classes of chemical compounds that react with the validated target gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

The present inventors have found that *Magnaporthe grisea* that cannot synthesize their own L-leucine are non-pathogenic on their host organism. To date there do not appear to be any publications demonstrating an anti-pathogenic effect of the knock-out, over-expression, antisense expression, or inhibition of the genes or gene products

involved in L-leucine biosynthesis in filamentous fungi. Thus, it has not been shown that the *de novo* biosynthesis of L-leucine is essential for fungal pathogenicity. And, thus, it would be desirable to determine the utility of the enzymes involved in L-leucine biosynthesis for evaluating antibiotic compounds, especially fungicides. If a fungal biochemical pathway or specific gene product in that pathway is shown to be required for fungal pathogenicity, various formats of *in vitro* and *in vivo* screening assays may be put in place to discover classes of chemical compounds that react with the validated target gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

The present inventors have found that *Magnaporthe grisea* that cannot synthesize their own L-threonine are non-pathogenic on their host organism. To date there do not appear to be any publications demonstrating an anti-pathogenic effect of the knock-out, over-expression, antisense expression, or inhibition of the genes or gene products involved in L-threonine biosynthesis in filamentous fungi. Thus, it has not been shown that the *de novo* biosynthesis of L-threonine is essential for fungal pathogenicity. Thus, it would be desirable to determine the utility of the enzymes involved in L-threonine biosynthesis for evaluating antibiotic compounds, especially fungicides. If a fungal biochemical pathway or specific gene product in that pathway is shown to be required for fungal pathogenicity, various formats of *in vitro* and *in vivo* screening assays may be put in place to discover classes of chemical compounds that react with the validated target gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

SUMMARY OF THE INVENTION

The present inventors have discovered that *in vivo* disruption of the genes encoding Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase in *Magnaporthe grisea* prevents or inhibits the pathogenicity of the fungus. Thus, the present inventors have discovered that Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase and Threonine synthase are essential for normal rice blast

pathogenicity, and can be used as targets for the identification of antibiotics, preferably fungicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably fungicides.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the reaction performed by Asparagine Synthase (ASN1) reaction. The Substrates/Products are L-aspartate, L-glutamine, and ATP and the Products/Substrates are L-asparagine, L-glutamate, AMP, and pyrophosphate. The function of the Asparagine Synthase enzyme is the interconversion of L-aspartate, L-glutamine, and ATP to L-asparagine, L-glutamate, AMP, and pyrophosphate. This reaction is part of the L-asparagine biosynthesis pathway.

Figure 2 shows a digital image showing the effect of ASN1 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type and the transposon insertion strains, KO1-2 and KO1-8. Leaf segments were imaged at five days post-inoculation.

Figure 3A&B. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-2 and KO1-8, were grown in (A) minimal media and (B) minimal media with the addition of L-asparagine, respectively. The x-axis shows time in days and the y-axis shows turbidity measured at 490 nanometers and 750 nanometers. The symbols represent wildtype (--♦--), transposon strain KO1-2 (--■--), and transposon strain KO1-8 (--▲--).

Figure 4 shows the reaction performed by 5-Aminolevulinate synthase (ALAS1) reaction. The Substrates/Products are succinyl-CoA and glycine and the Products/Substrates are 5-aminolevulinate, CoA, and CO_2. The function of the 5-Aminolevulinate synthase enzyme is the interconversion of succinyl-CoA and glycine to 5-aminolevulinate, CoA, and CO_2. This reaction is part of the heme biosynthesis pathway.

Figure 5 shows a digital image showing the effect of ALAS1 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type and the transposon insertion strains, KO1-1 and KO1-106. Leaf segments were imaged at five days post-inoculation.

Figure 6A&B. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-1 and KO1-106, were grown in (A) minimal media and (B) minimal media with the addition of 5-aminolevulinate, respectively. The x-axis shows time in days and the y-axis shows turbidity measured at 490 nanometers and 750 nanometers. The symbols represent wildtype (--♦--), transposon strain KO1-1 (--■--), and transposon strain KO1-106 (--▲--).

Figure 7 shows the reaction performed by histidinol-phosphatase (HISP1) reaction. The Substrates/Products are L-histidinol phosphate and H_2O and the Products/Substrates are L-histidinol and orthophosphate. The function of the histidinol-phosphatase enzyme is the interconversion of L-histidinol phosphate and H_2O to L-histidinol and orthophosphate. This reaction is part of the L-histidine biosynthesis pathway.

Figure 8 shows a digital image showing the effect of HISP1 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type and the transposon insertion strains, KO1-1 and KO1-3. Leaf segments were imaged at five days post-inoculation.

Figure 9A&B. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-1 and KO1-3, were grown in (A) minimal media and (B) minimal media with the addition of L-histidine, respectively. The x-axis shows time in days and the y-axis shows turbidity measured at 490 nanometers and 750 nanometers. The symbols represent wildtype (--♦--), transposon strain KO1-1
(--■--), and transposon strain KO1-3 (--▲--).

Figure 10 shows the reaction performed by 3-Isopropylmalate dehydratase (IPMD1) reaction. The Substrates/Products are 2-Isopropylmalate and H_2O and the Product/Substrate is 3-Isopropylmalate. The function of the 3-Isopropylmalate

dehydratase enzyme is the interconversion of 2-Isopropylmalate and H_2O to 3-Isopropylmalate. This reaction is part of the L-leucine biosynthesis pathway.

Figure 11 shows a digital image showing the effect of IPMD1 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type and the transposon insertion strains, KO1-3 and KO1-7. Leaf segments were imaged at five days post-inoculation.

Figure 12A&B. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-3 and KO1-7, were grown in (A) minimal media and (B) minimal media with the addition of L-leucine, respectively. The x-axis shows time in days and the y-axis shows turbidity measured at 490 nanometers and 750 nanometers. The symbols represent wildtype (--♦--), transposon strain KO1-3 (T1) (--■--), and transposon strain KO1-7 (T2) (--▲--).

Figure 13 shows the reaction performed by Threonine synthase (THR4) reaction. The Substrates/Products are O-phospho-L-homoserine and water and the Products/Substrates are L-threonine and orthophosphate. The function of the Threonine synthase enzyme is the interconversion of O-phospho-L-homoserine and water to L-threonine and orthophosphate. This reaction is part of the L-threonine biosynthesis pathway.

Figure 14 shows a digital image showing the effect of THR4 gene disruption on *Magnaporthe grisea* pathogenicity using whole plant infection assays. Rice variety CO39 was inoculated with wild-type and the transposon insertion strains, KO1-3 and KO1-22. Leaf segments were imaged at five days post-inoculation.

Figure 15A&B. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-3 and KO1-22, were grown in (A) minimal media and (B) minimal media with the addition of L-threonine, respectively. The x-axis shows time in days and the y-axis shows turbidity measured at 490 nanometers and 750 nanometers. The symbols represent wildtype (--♦--), transposon strain KO1-3 (--■--), and transposon strain KO1-22 (--▲--).

DETAILED DESCRIPTION OF THE INVENTION

Unless otherwise indicated, the following terms are intended to have the following meanings in interpreting the present invention.

The term "active against" in the context of compounds, agents, or compositions having antibiotic activity indicates that the compound exerts an effect on a particular target or targets which is deleterious to the *in vitro* and/or *in vivo* growth of an organism having that target or targets. In particular, a compound active against a gene exerts an action on a target which affects an expression product of that gene. This does not necessarily mean that the compound acts directly on the expression product of the gene, but instead indicates that the compound affects the expression product in a deleterious manner. Thus, the direct target of the compound may be, for example, at an upstream component which reduces transcription from the gene, resulting in a lower level of expression. Likewise, the compound may affect the level of translation of a polypeptide expression product, or may act on a downstream component of a biochemical pathway in which the expression product of the gene has a major biological role. Consequently, such a compound can be said to be active against the gene, against the gene product, or against the related component either upstream or downstream of that gene or expression product. While the term "active against" encompasses a broad range of potential activities, it also implies some degree of specificity of target. Therefore, for example, a general protease is not "active against" a particular gene which produces a polypeptide product. In contrast, a compound which inhibits a particular enzyme is active against that enzyme and against the gene which codes for that enzyme.

As used herein, the term "allele" refers to any of the alternative forms of a gene that may occur at a given locus.

The term "antibiotic" refers to any substance or compound that when contacted with a living cell, organism, virus, or other entity capable of replication, results in a reduction of growth, viability, or pathogenicity of that entity.

As used herein, the term "ALAS1" means a gene encoding 5-Aminolevulinate synthase activity, referring to an enzyme that catalyses the interconversion of succinyl-

CoA and glycine with 5-aminolevulinate, CoA, and CO_2, and may also be used to refer to the gene product.

As used herein, the terms "5-Aminolevulinate synthase" (EC 2.3.1.37) and "5-Aminolevulinate synthase polypeptide" are synonymous with "the ALAS1 gene product" and refer to an enzyme that catalyses the interconversion of succinyl-CoA and glycine with 5-aminolevulinate, CoA, and CO_2.

As used herein, the term "ASN1" means a gene encoding Asparagine Synthase activity, referring to an enzyme that catalyses the interconversion of L-aspartate, L-glutamine, and ATP with L-asparagine, L-glutamate, AMP, and pyrophosphate, and may also be used to refer to the gene product.

As used herein, the terms "Asparagine Synthase" (EC 6.3.5.4) and "Asparagine Synthase polypeptide" are synonymous with "the ASN1 gene product" and refer to an enzyme that catalyses the interconversion of L-aspartate, L-glutamine, and ATP with L-asparagine, L-glutamate, AMP, and pyrophosphate.

The term "binding" refers to a non-covalent or a covalent interaction, preferably non-covalent, that holds two molecules together. For example, two such molecules could be an enzyme and an inhibitor of that enzyme. Non-covalent interactions include hydrogen bonding, ionic interactions among charged groups, van der Waals interactions and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

The term "biochemical pathway" or "pathway" refers to a connected series of biochemical reactions normally occurring in a cell, or more broadly a cellular event such as cellular division or DNA replication. Typically, the steps in such a biochemical pathway act in a coordinated fashion to produce a specific product or products or to produce some other particular biochemical action. Such a biochemical pathway requires the expression product of a gene if the absence of that expression product either directly or indirectly prevents the completion of one or more steps in that pathway, thereby preventing or significantly reducing the production of one or more normal products or effects of that pathway. Thus, an agent specifically inhibits such a biochemical pathway requiring the expression product of a particular gene if the presence of the agent stops or substantially reduces the completion of the series of steps in that pathway. Such an agent,

may, but does not necessarily, act directly on the expression product of that particular gene.

As used herein, the term "cDNA" means complementary deoxyribonucleic acid.

As used herein, the term "CoA" means coenzyme A.

As used herein, the term "conditional lethal" refers to a mutation permitting growth and/or survival only under special growth or environmental conditions.

As used herein, the term "cosmid" refers to a hybrid vector, used in gene cloning, that includes a cos site (from the lambda bacteriophage). It also contains drug resistance marker genes and other plasmid genes. Cosmids are especially suitable for cloning large genes or multigene fragments.

As used herein, the term "dominant allele" refers to a dominant mutant allele in which a discernable mutant phenotype can be detected when this mutation is present in an organism that also contains a wild type (non-mutant), recessive allele, or other dominant allele.

As used herein, the term "DNA" means deoxyribonucleic acid.

As used herein, the term "ELISA" means enzyme-linked immunosorbent assay.

"Fungi" (singular: fungus) refers to whole fungi, fungal organs and tissues (*e.g.*, asci, hyphae, pseudohyphae, rhizoid, sclerotia, sterigmata, spores, sporodochia, sporangia, synnemata, conidia, ascostroma, cleistothecia, mycelia, perithecia, basidia and the like), spores, fungal cells and the progeny thereof. Fungi are a group of organisms (about 50,000 known species), including, but not limited to, mushrooms, mildews, moulds, yeasts, *etc.*, comprising the kingdom Fungi. They can either exist as single cells or make up a multicellular body called a mycelium, which consists of filaments known as hyphae. Most fungal cells are multinucleate and have cell walls, composed chiefly of chitin. Fungi exist primarily in damp situations on land and, because of the absence of chlorophyll and thus the inability to manufacture their own food by photosynthesis, are either parasites on other organisms or saprotrophs feeding on dead organic matter. The principal criteria used in classification are the nature of the spores produced and the presence or absence of cross walls within the hyphae. Fungi are distributed worldwide in terrestrial, freshwater, and marine habitats. Some live in the soil. Many pathogenic fungi cause disease in animals and man or in plants, while some saprotrophs are destructive to

timber, textiles, and other materials. Some fungi form associations with other organisms, most notably with algae to form lichens.

As used herein, the term "fungicide", "antifungal", or "antimycotic" refers to an antibiotic substance or compound that kills or suppresses the growth, viability, or pathogenicity of at least one fungus, fungal cell, fungal tissue or spore.

In the context of this disclosure, "gene" should be understood to refer to a unit of heredity. Each gene is composed of a linear chain of deoxyribonucleotides which can be referred to by the sequence of nucleotides forming the chain. Thus, "sequence" is used to indicate both the ordered listing of the nucleotides which form the chain, and the chain, itself, which has that sequence of nucleotides. ("Sequence" is used in the similar way in referring to RNA chains, linear chains made of ribonucleotides). The gene may include regulatory and control sequences, sequences which can be transcribed into an RNA molecule, and may contain sequences with unknown function. The majority of the RNA transcription products are messenger RNAs (mRNAs), which include sequences which are translated into polypeptides and may include sequences which are not translated. It should be recognized that small differences in nucleotide sequence for the same gene can exist between different fungal strains, or even within a particular fungal strain, without altering the identity of the gene.

As used in this disclosure, the terms "growth" or "cell growth" of an organism refers to an increase in mass, density, or number of cells of said organism. Some common methods for the measurement of growth include the determination of the optical density of a cell suspension, the counting of the number of cells in a fixed volume, the counting of the number of cells by measurement of cell division, the measurement of cellular mass or cellular volume, and the like.

As used in this disclosure, the term "growth conditional phenotype" indicates that a fungal strain having such a phenotype exhibits a significantly greater difference in growth rates in response to a change in one or more of the culture parameters than an otherwise similar strain not having a growth conditional phenotype. Typically, a growth conditional phenotype is described with respect to a single growth culture parameter, such as temperature. Thus, a temperature (or heat-sensitive) mutant (*i.e.*, a fungal strain having a heat-sensitive phenotype) exhibits significantly different growth, and preferably

no growth, under non-permissive temperature conditions as compared to growth under permissive conditions. In addition, such mutants preferably also show intermediate growth rates at intermediate, or semi-permissive, temperatures. Similar responses also result from the appropriate growth changes for other types of growth conditional phenotypes.

As used herein, the term "H_2O" means water.

As used herein, the term "heterologous ALAS1 gene" means a gene, not derived from *Magnaporthe grisea*, and having: at least 50% sequence identity, preferably 60%, 70%, 80%, 90%, 95%, 99% sequence identity and each integer unit of sequence identity from 50-100% in ascending order to SEQ ID NO: 4 or SEQ ID NO: 5; or at least 10% of the activity of a *Magnaporthe grisea* 5-Aminolevulinate synthase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

As used herein, the term "heterologous ASN1 gene" means a gene, not derived from *Magnaporthe grisea*, and having: at least 50% sequence identity, preferably 60%, 70%, 80%, 90%, 95%, 99% sequence identity and each integer unit of sequence identity from 50-100% in ascending order to SEQ ID NO: 1 or SEQ ID NO: 2; or at least 10% of the activity of a *Magnaporthe grisea* Asparagine Synthase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

As used herein, the term "heterologous HISP1 gene" means a gene, not derived from *Magnaporthe grisea*, and having: at least 50% sequence identity, preferably 60%, 70%, 80%, 90%, 95%, 99% sequence identity and each integer unit of sequence identity from 50-100% in ascending order to SEQ ID NO: 7 or SEQ ID NO: 8; or at least 10% of the activity of a *Magnaporthe grisea* histidinol-phosphatase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

As used herein, the terms "histidinol-phosphatase" (EC 3.1.3.15) and "histidinol-phosphatase polypeptide" are synonymous with "the HISP1 gene product" and refer to an enzyme that catalyses the interconversion of L-histidinol phosphate and H_2O with L-histidinol and orthophosphate.

As used herein, the term "heterologous IPMD1 gene" means a gene, not derived from *Magnaporthe grisea*, and having: at least 50% sequence identity, preferably 60%, 70%, 80%, 90%, 95%, 99% sequence identity and each integer unit of sequence identity

from 50-100% in ascending order to SEQ ID NO: 10 or SEQ ID NO: 11; or at least 10% of the activity of a *Magnaporthe grisea* 3-Isopropylmalate dehydratase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

As used herein, the term "heterologous THR4 gene" means a gene, not derived from *Magnaporthe grisea*, and having: at least 50% sequence identity, preferably 60%, 70%, 80%, 90%, 95%, 99% sequence identity and each integer unit of sequence identity from 50-100% in ascending order to SEQ ID NO: 13 or SEQ ID NO: 14; or at least 10% of the activity of a *Magnaporthe grisea* Threonine synthase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

As used herein, the term "HISP1" means a gene encoding histidinol-phosphatase activity, referring to an enzyme that catalyses the interconversion of L-histidinol phosphate and H_2O with L-histidinol and orthophosphate, and may also be used to refer to the gene product.

As used herein, the term "His-Tag" refers to an encoded polypeptide consisting of multiple consecutive histidine amino acids.

As used herein, the term "HPLC" means high pressure liquid chromatography.

As used herein, the terms "hph", "hygromycin B phosphotransferase", and "hygromycin resistance gene" refer to the *E. coli* hygromycin phosphotransferase gene or gene product.

As used herein, the term "hygromycin B" refers to an aminoglycosidic antibiotic, used for selection and maintenance of eukaryotic cells containing the *E. coli* hygromycin resistance gene.

"Hypersensitive" refers to a phenotype in which cells are more sensitive to antibiotic compounds than are wild-type cells of similar or identical genetic background.

"Hyposensitive" refers to a phenotype in which cells are less sensitive to antibiotic compounds than are wild-type cells of similar or identical genetic background.

As used herein, the term "imperfect state" refers to a classification of a fungal organism having no demonstrable sexual life stage.

The term "inhibitor", as used herein, refers to a chemical substance that inactivates the enzymatic activity or substantially reduces the level of enzymatic activity,

of any one of Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction by 50%, more preferably a reduction of at least one magnitude, i.e. to 10%. The inhibitor may function by interacting directly with the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

A polynucleotide may be "introduced" into a fungal cell by any means known to those of skill in the art, including transfection, transformation or transduction, transposable element, electroporation, particle bombardment, infection and the like. The introduced polynucleotide may be maintained in the cell stably if it is incorporated into a non-chromosomal autonomous replicon or integrated into the fungal chromosome. Alternatively, the introduced polynucleotide may be present on an extra-chromosomal non-replicating vector and be transiently expressed or transiently active.

As used herein, the term "IPMD1" means a gene encoding 3-Isopropylmalate dehydratase activity, referring to an enzyme that catalyses the interconversion of 2-Isopropylmalate and H_2O with 3-Isopropylmalate, and may also refer to the gene product.

As used herein, the terms "3-Isopropylmalate dehydratase" (EC 4.2.1.33), "α-isopropylmalate isomerase" and "3-Isopropylmalate dehydratase polypeptide" are synonymous with "the IPMD1 gene product" and refer to an enzyme that catalyses the interconversion of 2-Isopropylmalate and H_2O with 3-Isopropylmalate.

As used herein, the term "knockout" or "gene disruption" refers to the creation of organisms carrying a null mutation (a mutation in which there is no active gene product), a partial null mutation or mutations, or an alteration or alterations in gene regulation by interrupting a DNA sequence through insertion of a foreign piece of DNA. Usually the foreign DNA encodes a selectable marker.

As used herein, the term "LB agar" means Luria's Broth agar.

The term "method of screening" means that the method is suitable, and is typically used, for testing for a particular property or effect in a large number of compounds. Typically, more than one compound is tested simultaneously (as in a

96-well microtiter plate), and preferably significant portions of the procedure can be automated. "Method of screening" also refers to the determination of a set of different properties or effects of one compound simultaneously.

As used herein, the term "mRNA" means messenger ribonucleic acid.

As used herein, the term "mutant form" of a gene refers to a gene which has been altered, either naturally or artificially, changing the base sequence of the gene. The change in the base sequence may be of several different types, including changes of one or more bases for different bases, deletions, and/or insertions, such as by a transposon. By contrast, a normal form of a gene (wild type) is a form commonly found in natural populations of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

As used herein, the term "Ni" refers to nickel.

As used herein, the term "Ni-NTA" refers to nickel sepharose.

As used herein, a "normal" form of a gene (wild type) is a form commonly found in natural populations of an organism. Commonly a single form of a gene will predominate in natural populations. In general, such a gene is suitable as a normal form of a gene, however, other forms which provide similar functional characteristics may also be used as a normal gene. In particular, a normal form of a gene does not confer a growth conditional phenotype on the strain having that gene, while a mutant form of a gene suitable for use in these methods does provide such a growth conditional phenotype.

As used herein, the term "one form" of a gene is synonymous with the term "gene", and a "different form" of a gene refers to a gene that has greater than 49% sequence identity and less than 100% sequence identity with said first form.

As used herein, the term "pathogenicity" refers to a capability of causing disease. The term is applied to parasitic microorganisms in relation to their hosts.

As used herein, the term "PCR" means polymerase chain reaction.

The "percent (%) sequence identity" between two polynucleotide or two polypeptide sequences is determined according to the either the BLAST program (Basic Local Alignment Search Tool; (Altschul, S.F., W. Gish, *et al.* (1990) J Mol Biol *215*: 403 - 10 (PMID: 2231712)) at the National Center for Biotechnology or using Smith Waterman Alignment (Smith, T. F. and M. S. Waterman (1981) J Mol Biol *147*: 195 - 7 (PMID: 7265238)) as incorporated into GeneMatcher Plus™. It is understood that for the purposes of determining sequence identity when comparing a DNA sequence to an RNA sequence, a thymine nucleotide is equivalent to a uracil nucleotide.

By "polypeptide" is meant a chain of at least two amino acids joined by peptide bonds. The chain may be linear, branched, circular or combinations thereof. Preferably, polypeptides are from about 10 to about 1000 amino acids in length, more preferably 10-50 amino acids in length. The polypeptides may contain amino acid analogs and other modifications, including, but not limited to glycosylated or phosphorylated residues.

As used herein, the term "proliferation" is synonymous to the term "growth".

As used herein, the term "reverse transcriptase-PCR" means reverse transcription-polymerase chain reaction.

As used herein, the term "RNA" means ribonucleic acid.

As used herein, "semi-permissive conditions" are conditions in which the relevant culture parameter for a particular growth conditional phenotype is intermediate between permissive conditions and non-permissive conditions. Consequently, in semi-permissive conditions an organism having a growth conditional phenotype will exhibit growth rates intermediate between those shown in permissive conditions and non-permissive conditions. In general, such intermediate growth rate may be due to a mutant cellular component which is partially functional under semi-permissive conditions, essentially fully functional under permissive conditions, and is non-functional or has very low function under non-permissive conditions, where the level of function of that component is related to the growth rate of the organism. An intermediate growth rate may also be a result of a nutrient substance or substances that are present in amounts not sufficient for optimal growth rates to be achieved.

"Sensitivity phenotype" refers to a phenotype that exhibits either hypersensitivity or hyposensitivity.

The term "specific binding" refers to an interaction between any one of Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence and/or the conformation of the Asparagine Synthase, 5-Aminolevulinate synthase, histidinol-phosphatase, 3-Isopropylmalate dehydratase or Threonine synthase.

As used herein, the term "THR4" means a gene encoding Threonine synthase activity, referring to an enzyme that catalyses the interconversion of O-phospho-L-homoserine and water with L-threonine and orthophosphate, and may also be used to refer to the gene product.

As used herein, the terms "Threonine synthase" (EC 4.2.99.2) and "Threonine synthase polypeptide" are synonymous with "the THR4 gene product" and refer to an enzyme that catalyses the interconversion of O-phospho-L-homoserine and water with L-threonine and orthophosphate.

As used herein, the term "TLC" means thin layer chromatography.

"Transform", as used herein, refers to the introduction of a polynucleotide (single or double stranded DNA, RNA, or a combination thereof) into a living cell by any means. Transformation may be accomplished by a variety of methods, including, but not limited to, electroporation, polyethylene glycol mediated uptake, particle bombardment, agrotransformation, and the like. This process may result in transient or stable expression of the transformed polynucleotide. By "stably transformed" is meant that the sequence of interest is integrated into a replicon in the cell, such as a chromosome or episome. Transformed cells encompass not only the end product of a transformation process, but also the progeny thereof which retain the polynucleotide of interest.

For the purposes of the invention, "transgenic" refers to any cell, spore, tissue or part, that contains all or part of at least one recombinant polynucleotide. In many cases, all or part of the recombinant polynucleotide is stably integrated into a chromosome or stable extra-chromosomal element, so that it is passed on to successive generations.

As used herein, the term "transposase" refers to an enzyme that catalyzes transposition. Preferred transposons are described in WO 00/55346, PCT/US00/07317, and US 09/658859.

As used herein, the term "transposition" refers to a complex genetic rearrangement process involving the movement or copying of a polynucleotide (transposon) from one location and insertion into another, often within or between a genome or genomes, or DNA constructs such as plasmids, bacmids, and cosmids.

As used herein, the term "transposon" (also known as a "transposable element", "transposable genetic element", "mobile element", or "jumping gene") refers to a mobile DNA element such as those, for example, described in WO 00/55346, PCT/US00/07317, and US 09/658859. Transposons can disrupt gene expression or cause deletions and inversions, and hence affect both the genotype and phenotype of the organisms concerned. The mobility of transposable elements has long been used in genetic manipulation, to introduce genes or other information into the genome of certain model systems.

As used herein, the term "Tween 20" means sorbitan mono-9-octadecenoate poly(oxy-1,1-ethanediyl).

As used in this disclosure, the term "viability" of an organism refers to the ability of an organism to demonstrate growth under conditions appropriate for said organism, or to demonstrate an active cellular function. Some examples of active cellular functions include respiration as measured by gas evolution, secretion of proteins and/or other compounds, dye exclusion, mobility, dye oxidation, dye reduction, pigment production, changes in medium acidity, and the like.

The present inventors have discovered that disruption of the ASN1 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that Asparagine Synthase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit ASN1 gene expression or biological activity of its gene product(s). Such methods include ligand binding assays, assays for enzyme activity, cell-based assays, and assays for ASN1 gene expression. Any compound that is a ligand for Asparagine Synthase may have antibiotic activity. For the purposes of the invention, "ligand" refers to a molecule

that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting an Asparagine Synthase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said Asparagine Synthase polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

The Asparagine Synthase protein may have the amino acid sequence of a naturally occurring Asparagine Synthase found in a fungus, animal, plant, or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the Asparagine Synthase is a fungal Asparagine Synthase. The cDNA (SEQ ID NO: 1) encoding the Asparagine Synthase protein, the genomic DNA (SEQ ID NO: 2) encoding the *M. grisea* protein, and the polypeptide (SEQ ID NO: 3) can be found herein.

In one aspect, the invention also provides for a polypeptide consisting essentially of SEQ ID NO: 3. For the purposes of the invention, a polypeptide consisting essentially of SEQ ID NO: 3 has at least 80% sequence identity with SEQ ID NO: 3 and catalyses the interconversion of L-aspartate, L-glutamine, and ATP with L-asparagine, L-glutamate, AMP, and pyrophosphate with at least 10% of the activity of SEQ ID NO: 3. Preferably, the polypeptide consisting essentially of SEQ ID NO: 3 has at least 85% sequence identity with SEQ ID NO: 3, more preferably the sequence identity is at least 90%, most preferably the sequence identity is at least 95% or 97 or 99%, or any integer from 80-100% sequence identity in ascending order. And, preferably, the polypeptide consisting essentially of SEQ ID NO: 3 has at least 25%, at least 50%, at least 75% or at least 90% of the activity of *M. grisea* Asparagine Synthase, or any integer from 60-100% activity in ascending order.

By "fungal Asparagine Synthase" is meant an enzyme that can be found in at least one fungus, and which catalyzes the interconversion of L-aspartate, L-glutamine, and ATP with L-asparagine, L-glutamate, AMP, and pyrophosphate. The Asparagine

Synthase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

In one embodiment, the Asparagine Synthase is a *Magnaporthe* Asparagine Synthase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* Asparagine Synthase is from *Magnaporthe grisea*.

In various embodiments, the Asparagine Synthase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of an Asparagine Synthase polypeptide may be used in the methods of the invention, preferably if the fragments include an intact or nearly intact epitope that occurs on the biologically active wildtype Asparagine Synthase. The fragments comprise at least 10 consecutive amino acids of an Asparagine Synthase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, or at least 580 consecutive amino acids residues of an

Asparagine Synthase. In one embodiment, the fragment is from a *Magnaporthe* Asparagine Synthase. Preferably, the fragment contains an amino acid sequence conserved among fungal Asparagine Synthases.

Polypeptides having at least 50% sequence identity with a fungal Asparagine Synthase are also useful in the methods of the invention. Preferably, the sequence identity is at least 60%, more preferably the sequence identity is at least 70%, most preferably the sequence identity is at least 80% or 90 or 95 or 99%, or any integer from 60-100% sequence identity in ascending order.

In addition, it is preferred that the polypeptide has at least 10% of the activity of a fungal Asparagine Synthase. More preferably, the polypeptide has at least 25%, at least 50%, at least 75% or at least 90% of the activity of a fungal Asparagine Synthase. Most preferably, the polypeptide has at least 10%, at least 25%, at least 50%, at least 75% or at least 90% of the activity of the *M. grisea* Asparagine Synthase protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal Asparagine Synthase; a polypeptide having at least 50% sequence identity with a fungal Asparagine Synthase; and a polypeptide having at least 10% of the activity of a fungal Asparagine Synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with an Asparagine Synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound Asparagine Synthase is

detected. In a preferred embodiment, bound Asparagine Synthase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, Asparagine Synthase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit Asparagine Synthase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with said antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of said fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit Asparagine Synthase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. Asparagine Synthase catalyzes the irreversible or reversible reaction L-aspartate, L-glutamine, and ATP = L-asparagine, L-glutamate, AMP, and pyrophosphate (see Figure 1). Methods for detection of L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-aspartate, L-glutamine, and ATP with an Asparagine Synthase;

b) contacting L-aspartate, L-glutamine, and ATP with Asparagine Synthase and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with an Asparagine Synthase;

b) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with an Asparagine Synthase and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

Enzymatically active fragments of a fungal Asparagine Synthase are also useful in the methods of the invention. For example, an enzymatically active polypeptide

comprising at least 100 consecutive amino acid residues of a fungal Asparagine Synthase may be used in the methods of the invention. In addition, an enzymatically active polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95% or at least 98% sequence identity with a fungal Asparagine Synthase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal Asparagine Synthase and at least 10%, 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-aspartate, L-glutamine, and ATP with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with an Asparagine Synthase, a polypeptide having at least 50% sequence identity with an Asparagine Synthase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an Asparagine Synthase;

b) contacting L-aspartate, L-glutamine, and ATP with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with an Asparagine Synthase, a polypeptide having at least 50% sequence identity with an Asparagine Synthase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an Asparagine Synthase;

b) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate, with a polypeptide and said test compound; and

c) determining the change in concentration for at least one of the following, L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

For the *in vitro* enzymatic assays, Asparagine Synthase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of Asparagine Synthase may be described in Van Heeke and Schuster (1989) J Biol Chem *264*: 5503 – 9 (PMID: 2564390). Other methods for the purification of Asparagine Synthase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of an Asparagine Synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said Asparagine Synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of Asparagine Synthase in steps (a) and (b), wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

Expression of Asparagine Synthase can be measured by detecting the ASN1 primary transcript or mRNA, Asparagine Synthase polypeptide, or Asparagine Synthase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology* Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for detecting ASN1 RNA

include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using an ASN1 promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect ASN1 protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with ASN1, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators, preferably inhibitors, of ASN1 expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato

Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided is a method of screening for an antibiotic by determining whether a test compound is active against the gene identified (SEQ ID NO: 1 or SEQ ID NO: 2), its gene product (SEQ ID NO: 3), or the biochemical pathway or pathways on which it functions.

In one particular embodiment, the method is performed by providing an organism having a first form of the gene corresponding to either SEQ ID NO: 1 or SEQ ID NO: 2, either a normal form, a mutant form, a homologue, or a heterologous ASN1 gene that performs a similar function as ASN1. The first form of ASN1 may or may not confer a growth conditional phenotype, *i.e.*, a L-asparagine requiring phenotype, and/or a hypersensitivity or hyposensitivity phenotype on the organism having that altered form. In one particular embodiment a mutant form contains a transposon insertion. A comparison organism having a second form of an ASN1, different from the first form of the gene is also provided, and the two organisms are separately contacted with a test compound. The growth of the two organisms in the presence of the test compound is then compared.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of an Asparagine Synthase gene, and providing comparison cells having a different form of an Asparagine Synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and said comparison cells in the presence of the test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that the optional determination of the growth of said first organism and said comparison second organism in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different genes. It is also recognized that any combination of two different forms of an ASN1 gene, including normal genes, mutant genes, homologues, and functional homologues may be used in this method. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment the organism is *Magnaporthe grisea.*

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry, New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which ASN1 functions, comprising:

a) providing cells having one form of a gene in the L-asparagine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;
b) contacting said cells and said comparison cells with a test compound; and
c) determining the growth of said cells and said comparison cells in the presence of said test compound, wherein a difference in growth between said cells and said

comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger et al. (1993) Principles of Biochemistry).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which ASN1 functions, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-asparagine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism, wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that determination of the growth of said organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The present inventors have discovered that disruption of the ALAS1 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that 5-Aminolevulinate synthase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit ALAS1 gene expression or biological activity of its gene product(s). Such methods include ligand binding assays, assays for enzyme activity, cell-based assays, and assays for ALAS1 gene expression. Any compound that is a ligand for 5-Aminolevulinate synthase may have antibiotic activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a 5-Aminolevulinate synthase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said 5-Aminolevulinate synthase polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

The 5-Aminolevulinate synthase protein may have the amino acid sequence of a naturally occurring 5-Aminolevulinate synthase found in a fungus, animal, plant, or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the 5-Aminolevulinate synthase is a fungal 5-Aminolevulinate synthase. The cDNA (SEQ ID NO: 4) encoding the *M. grisea* 5-Aminolevulinate synthase protein, the genomic DNA (SEQ ID NO: 5) encoding the protein, and the polypeptide (SEQ ID NO: 6) can be found herein.

In one aspect, the invention also provides for a polypeptide consisting essentially of SEQ ID NO: 6. For the purposes of the invention, a polypeptide consisting essentially of SEQ ID NO: 6 has at least 80% sequence identity with SEQ ID NO: 6 and catalyses the interconversion of succinyl-CoA and glycine with 5-aminolevulinate, CoA, and CO_2 with at least 10% of the activity of SEQ ID NO: 6. Preferably, the polypeptide consisting essentially of SEQ ID NO: 6 has at least 85% sequence identity with SEQ ID NO: 6, more preferably the sequence identity is at least 90%, most preferably the sequence identity is at least 95% or 97 or 99%, or any integer from 80-100% sequence identity in ascending order. And, preferably, the polypeptide consisting essentially of SEQ ID NO: 6 has at least 25%, at least 50%, at least 75% or at least 90% of the activity of *M. grisea* 5-Aminolevulinate synthase, or any integer from 60-100% activity in ascending order.

By "fungal 5-Aminolevulinate synthase" is meant an enzyme that can be found in at least one fungus, and which catalyzes the interconversion of succinyl-CoA and glycine with 5-aminolevulinate, CoA, and CO_2. The 5-Aminolevulinate synthase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

In one embodiment, the 5-Aminolevulinate synthase is a *Magnaporthe* 5-Aminolevulinate synthase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* 5-Aminolevulinate synthase is from *Magnaporthe grisea*.

In various embodiments, the 5-Aminolevulinate synthase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust

(*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of a 5-Aminolevulinate synthase polypeptide may be used in the methods of the invention, preferably if the fragments include an intact or nearly intact epitope that occurs on the biologically active wildtype 5-Aminolevulinate synthase. The fragments comprise at least 10 consecutive amino acids of a 5-Aminolevulinate synthase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, or at least 610 consecutive amino acids residues of a 5-Aminolevulinate synthase. In one embodiment, the fragment is from a *Magnaporthe* 5-Aminolevulinate synthase. Preferably, the fragment contains an amino acid sequence conserved among fungal 5-Aminolevulinate synthases.

Polypeptides having at least 50% sequence identity with a fungal 5-Aminolevulinate synthase are also useful in the methods of the invention. Preferably, the sequence identity is at least 60%, more preferably the sequence identity is at least 70%, most preferably the sequence identity is at least 80% or 90 or 95 or 99%, or any integer from 60-100% sequence identity in ascending order.

In addition, it is preferred that the polypeptide has at least 10% of the activity of a fungal 5-Aminolevulinate synthase. More preferably, the polypeptide has at least 25%, at least 50%, at least 75% or at least 90% of the activity of a fungal 5-Aminolevulinate synthase. Most preferably, the polypeptide has at least 10%, at least 25%, at least 50%, at least 75% or at least 90% of the activity of the *M. grisea* 5-Aminolevulinate synthase protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal 5-Aminolevulinate synthase; a polypeptide having at least 50% sequence identity with a fungal 5-Aminolevulinate synthase; and a polypeptide having at least 10% of the activity of a fungal 5-Aminolevulinate synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a 5-Aminolevulinate synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound 5-Aminolevulinate synthase is detected. In a preferred embodiment, bound 5-Aminolevulinate synthase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, 5-Aminolevulinate synthase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit 5-Aminolevulinate synthase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising:

contacting a fungus or fungal cells with said antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of said fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit 5-Aminolevulinate synthase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. 5-Aminolevulinate synthase catalyzes the irreversible or reversible reaction succinyl-CoA and glycine = 5-aminolevulinate, CoA, and CO_2 (see Figure 1). Methods for detection of succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting succinyl-CoA and glycine with a 5-Aminolevulinate synthase;

b) contacting succinyl-CoA and glycine with 5-Aminolevulinate synthase and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 5-aminolevulinate, CoA, and CO_2 with a 5-Aminolevulinate synthase;

b) contacting 5-aminolevulinate, CoA, and CO_2 with a 5-Aminolevulinate synthase and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

Enzymatically active fragments of a fungal 5-Aminolevulinate synthase are also useful in the methods of the invention. For example, an enzymatically active polypeptide comprising at least 100 consecutive amino acid residues of a fungal 5-Aminolevulinate synthase may be used in the methods of the invention. In addition, an enzymatically active polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95% or at least 98% sequence identity with a fungal 5-Aminolevulinate synthase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal 5-Aminolevulinate synthase and at least 10%, 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting succinyl-CoA and glycine with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase; a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase and having at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 5-Aminolevulinate synthase;

b) contacting succinyl-CoA and glycine with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2;

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 5-aminolevulinate, CoA, and CO_2 with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase; a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase and at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 5-Aminolevulinate synthase;

b) contacting 5-aminolevulinate, CoA, and CO_2, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following, succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2;

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

For the *in vitro* enzymatic assays, 5-Aminolevulinate synthase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of 5-Aminolevulinate synthase may be described in Volland and Felix (1984) Eur J Biochem *142*: 551 - 7 (PMID: 6381051). Other methods for the purification of 5-Aminolevulinate synthase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a 5-Aminolevulinate synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said 5-Aminolevulinate synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of 5-Aminolevulinate synthase in steps (a) and (b);

wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

Expression of 5-Aminolevulinate synthase can be measured by detecting the ALAS1 primary transcript or mRNA, 5-Aminolevulinate synthase polypeptide, or 5-Aminolevulinate synthase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology* Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for detecting ALAS1 RNA include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using an ALAS1 promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect ALAS1 protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with ALAS1, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators, preferably inhibitors, of ALAS1 expression or activity can then be used to

control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided is a method of screening for an antibiotic by determining whether a test compound is active against the gene identified (SEQ ID NO: 4 or SEQ ID NO: 5), its gene product (SEQ ID NO: 6), or the biochemical pathway or pathways it functions on.

In one particular embodiment, the method is performed by providing an organism having a first form of the gene corresponding to either SEQ ID NO: 4 or SEQ ID NO: 5, either a normal form, a mutant form, a homologue, or a heterologous ALAS1 gene that performs a similar function as ALAS1. The first form of ALAS1 may or may not confer a growth conditional phenotype, *i.e.*, a 5-aminolevulinate requiring phenotype, and/or a hypersensitivity or hyposensitivity phenotype on the organism having that altered form. In one particular embodiment a mutant form contains a transposon insertion. A comparison organism having a second form of an ALAS1, different from the first form of the gene is also provided, and the two organisms are separately contacted with a test compound. The growth of the two organisms in the presence of the test compound is then compared.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a 5-Aminolevulinate synthase gene, and providing comparison cells having a different form of a 5-Aminolevulinate synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and said comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that the optional determination of the growth of said first organism and said comparison second organism in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different genes. It is also recognized that any combination of two different forms of an ALAS1 gene, including normal genes, mutant genes, homologues, and functional

homologues may be used in this method. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which ALAS1 functions, comprising:

d) providing cells having one form of a gene in the heme biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;
e) contacting said cells and said comparison cells with a test compound; and
f) determining the growth of said cells and said comparison cells in the presence of said test compound;

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which ALAS1 functions, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of 5-aminolevulinate than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism;

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that determination of the growth of said organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The present inventors have discovered that disruption of the HISP1 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that histidinol-phosphatase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit HISP1 gene expression or biological activity of its gene product(s). Such methods include ligand binding assays, assays for enzyme activity, cell-based assays, and assays

for HISP1 gene expression. Any compound that is a ligand for histidinol-phosphatase may have antibiotic activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a histidinol-phosphatase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said histidinol-phosphatase polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

The histidinol-phosphatase protein may have the amino acid sequence of a naturally occurring histidinol-phosphatase found in a fungus, animal, plant, or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the histidinol-phosphatase is a fungal histidinol-phosphatase. The cDNA (SEQ ID NO: 7) encoding the histidinol-phosphatase protein, the genomic DNA (SEQ ID NO: 8) encoding the *M. grisea* protein, and the polypeptide (SEQ ID NO: 9) can be found herein.

In one aspect, the invention also provides for a polypeptide consisting essentially of SEQ ID NO: 9. For the purposes of the invention, a polypeptide consisting essentially of SEQ ID NO: 9 has at least 80% sequence identity with SEQ ID NO: 9 and catalyses the interconversion of L-histidinol phosphate and H_2O with L-histidinol and orthophosphate with at least 10% of the activity of SEQ ID NO: 9. Preferably, the polypeptide consisting essentially of SEQ ID NO: 9 has at least 85% sequence identity with SEQ ID NO: 9, more preferably the sequence identity is at least 90%, most preferably the sequence identity is at least 95% or 97 or 99%, or any integer from 80-100% sequence identity in ascending order. And, preferably, the polypeptide consisting essentially of SEQ ID NO: 9 has at least 25%, at least 50%, at least 75% or at least 90% of the activity of *M. grisea* histidinol-phosphatase, or any integer from 60-100% activity in ascending order.

By "fungal histidinol-phosphatase" is meant an enzyme that can be found in at least one fungus, and which catalyzes the interconversion of L-histidinol phosphate and H_2O with L-histidinol and orthophosphate. The histidinol-phosphatase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

In one embodiment, the histidinol-phosphatase is a *Magnaporthe* histidinol-phosphatase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* histidinol-phosphatase is from *Magnaporthe grisea*.

In various embodiments, the histidinol-phosphatase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of a histidinol-phosphatase polypeptide may be used in the methods of the invention, preferably if the fragments include an intact or nearly intact epitope that occurs on the biologically active wildtype histidinol-phosphatase. The fragments comprise at least 10 consecutive amino acids of a histidinol-phosphatase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130,

140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, or at least 330 consecutive amino acids residues of a histidinol-phosphatase. In one embodiment, the fragment is from a *Magnaporthe* histidinol-phosphatase. Preferably, the fragment contains an amino acid sequence conserved among fungal histidinol-phosphatases.

Polypeptides having at least 50% sequence identity with a fungal histidinol-phosphatase are also useful in the methods of the invention. Preferably, the sequence identity is at least 60%, more preferably the sequence identity is at least 70%, most preferably the sequence identity is at least 80% or 90 or 95 or 99%, or any integer from 60-100% sequence identity in ascending order.

In addition, it is preferred that the polypeptide has at least 10% of the activity of a fungal histidinol-phosphatase. More preferably, the polypeptide has at least 25%, at least 50%, at least 75% or at least 90% of the activity of a fungal histidinol-phosphatase. Most preferably, the polypeptide has at least 10%, at least 25%, at least 50%, at least 75% or at least 90% of the activity of the *M. grisea* histidinol-phosphatase protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal histidinol-phosphatase; a polypeptide having at least 50% sequence identity with a fungal histidinol-phosphatase; and a polypeptide having at least 10% of the activity of a fungal histidinol-phosphatase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For

example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a histidinol-phosphatase protein or a fragment or variant thereof, the unbound protein is removed and the bound histidinol-phosphatase is detected. In a preferred embodiment, bound histidinol-phosphatase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, histidinol-phosphatase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit histidinol-phosphatase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with said antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of said fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least

90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit histidinol-phosphatase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. Histidinol-phosphatase catalyzes the irreversible or reversible reaction L-histidinol phosphate and H_2O = L-histidinol and orthophosphate (see Figure 1). Methods for detection of L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol phosphate and H_2O with a histidinol-phosphatase;

b) contacting L-histidinol phosphate and H_2O with histidinol-phosphatase and said test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol and orthophosphate with a histidinol-phosphatase;

b) contacting L-histidinol and orthophosphate with a histidinol-phosphatase and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

Enzymatically active fragments of a fungal histidinol-phosphatase are also useful in the methods of the invention. For example, an enzymatically active polypeptide

comprising at least 100 consecutive amino acid residues of a fungal histidinol-phosphatase may be used in the methods of the invention. In addition, an enzymatically active polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95% or at least 98% sequence identity with a fungal histidinol-phosphatase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal histidinol-phosphatase and at least 10%, 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol phosphate and H_2O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a histidinol-phosphatase; a polypeptide having at least 50% sequence identity with a histidinol-phosphatase and having at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a histidinol-phosphatase;

b) contacting L-histidinol phosphate and H_2O with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate.

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol and orthophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a histidinol-phosphatase; a polypeptide having at least 50% sequence identity with a histidinol-phosphatase and at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a histidinol-phosphatase;

b) contacting L-histidinol and orthophosphate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following, L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate;

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

For the *in vitro* enzymatic assays, histidinol-phosphatase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of histidinol-phosphatase may be described in Millay and Houston (1973) Biochemistry *12*: 2591 – 2596 (PMID: 4351203). Other methods for the purification of histidinol-phosphatase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a histidinol-phosphatase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said histidinol-phosphatase in said cell, cells, tissue, or organism; and

c) comparing the expression of histidinol-phosphatase in steps (a) and (b);

wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

Expression of histidinol-phosphatase can be measured by detecting the HISP1 primary transcript or mRNA, histidinol-phosphatase polypeptide, or histidinol-phosphatase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology* Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for

detecting HISP1 RNA include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using a HISP1 promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect HISP1 protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with HISP1, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators, preferably inhibitors, of HISP1 expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato

Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided is a method of screening for an antibiotic by determining whether a test compound is active against the gene identified (SEQ ID NO: 7 or SEQ ID NO: 8), its gene product (SEQ ID NO: 9), or the biochemical pathway or pathways it functions on.

In one particular embodiment, the method is performed by providing an organism having a first form of the gene corresponding to either SEQ ID NO: 7 or SEQ ID NO: 8, either a normal form, a mutant form, a homologue, or a heterologous HISP1 gene that performs a similar function as HISP1. The first form of HISP1 may or may not confer a growth conditional phenotype, *i.e.*, a L-histidine requiring phenotype, and/or a hypersensitivity or hyposensitivity phenotype on the organism having that altered form. In one particular embodiment a mutant form contains a transposon insertion. A comparison organism having a second form of a HISP1, different from the first form of the gene is also provided, and the two organisms are separately contacted with a test compound. The growth of the two organisms in the presence of the test compound is then compared.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a histidinol-phosphatase gene, and providing comparison cells having a different form of a histidinol-phosphatase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and said comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that the optional determination of the growth of said first organism and said comparison second organism in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different genes. It is also recognized that any combination of two different forms of a HISP1 gene, including normal genes, mutant genes, homologues, and functional homologues may be used in this method. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which HISP1 functions, comprising:

g) providing cells having one form of a gene in the L-histidine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene.

h) contacting said cells and said comparison cells with a test compound; and

i) determining the growth of said cells and said comparison cells in the presence of said test compound;

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which HISP1 functions, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-histidine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism;

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that determination of the growth of said organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The present inventors have discovered that disruption of the IPMD1 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that 3-Isopropylmalate dehydratase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit IPMD1 gene expression or biological activity of its gene product(s). Such methods include ligand binding assays, assays for enzyme activity, cell-based assays, and assays for IPMD1 gene expression. Any compound that is a ligand for 3-Isopropylmalate dehydratase may have antibiotic activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a 3-Isopropylmalate dehydratase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said 3-Isopropylmalate dehydratase polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

The 3-Isopropylmalate dehydratase protein may have the amino acid sequence of a naturally occurring 3-Isopropylmalate dehydratase found in a fungus, animal, plant, or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the 3-Isopropylmalate dehydratase is a fungal 3-Isopropylmalate dehydratase. The cDNA (SEQ ID NO: 10) encoding the 3-Isopropylmalate dehydratase protein, the genomic DNA (SEQ ID NO: 11) encoding the *M. grisea* protein, and the polypeptide (SEQ ID NO: 12) can be found herein.

In one aspect, the invention also provides for a polypeptide consisting essentially of SEQ ID NO: 12. For the purposes of the invention, a polypeptide consisting essentially of SEQ ID NO: 12 has at least 80% sequence identity with SEQ ID NO: 12 and catalyses the interconversion of 2-Isopropylmalate and H_2O with 3-Isopropylmalate with at least 10% of the activity of SEQ ID NO: 12. Preferably, the polypeptide consisting essentially of SEQ ID NO: 12 has at least 85% sequence identity with SEQ ID NO: 12, more preferably the sequence identity is at least 90%, most preferably the sequence identity is at least 95% or 97 or 99%, or any integer from 80-100% sequence identity in ascending order. And, preferably, the polypeptide consisting essentially of SEQ ID NO: 12 has at least 25%, at least 50%, at least 75% or at least 90% of the activity of *M. grisea* 3-Isopropylmalate dehydratase, or any integer from 60-100% activity in ascending order.

By "fungal 3-Isopropylmalate dehydratase" is meant an enzyme that can be found in at least one fungus, and which catalyzes the interconversion of 2-Isopropylmalate and H_2O with 3-Isopropylmalate. The 3-Isopropylmalate dehydratase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

In one embodiment, the 3-Isopropylmalate dehydratase is a *Magnaporthe* 3-Isopropylmalate dehydratase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* 3-Isopropylmalate dehydratase is from *Magnaporthe grisea*.

In various embodiments, the 3-Isopropylmalate dehydratase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust

(*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of a 3-Isopropylmalate dehydratase polypeptide may be used in the methods of the invention, preferably if the fragments include an intact or nearly intact epitope that occurs on the biologically active wildtype 3-Isopropylmalate dehydratase. The fragments comprise at least 10 consecutive amino acids of a 3-Isopropylmalate dehydratase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, or at least 770 consecutive amino acids residues of a 3-Isopropylmalate dehydratase. In one embodiment, the fragment is from a *Magnaporthe* 3-Isopropylmalate dehydratase. Preferably, the fragment contains an amino acid sequence conserved among fungal 3-Isopropylmalate dehydratases.

Polypeptides having at least 50% sequence identity with a fungal 3-Isopropylmalate dehydratase are also useful in the methods of the invention. Preferably, the sequence identity is at least 60%, more preferably the sequence identity is at least 70%, most preferably the sequence identity is at least 80% or 90 or 95 or 99%, or any integer from 60-100% sequence identity in ascending order.

In addition, it is preferred that the polypeptide has at least 10% of the activity of a fungal 3-Isopropylmalate dehydratase. More preferably, the polypeptide has at least 25%, at least 50%, at least 75% or at least 90% of the activity of a fungal 3-Isopropylmalate dehydratase. Most preferably, the polypeptide has at least 10%, at least 25%, at least 50%, at least 75% or at least 90% of the activity of the *M. grisea* 3-Isopropylmalate dehydratase protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a fungal 3-Isopropylmalate dehydratase; and a polypeptide having at least 10% of the activity of a fungal 3-Isopropylmalate dehydratase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide;

wherein binding indicates that said test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a 3-Isopropylmalate dehydratase protein or a fragment or variant thereof, the unbound protein is removed and the bound 3-Isopropylmalate dehydratase is detected. In a preferred embodiment, bound 3-Isopropylmalate dehydratase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, 3-Isopropylmalate dehydratase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit 3-Isopropylmalate dehydratase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the

invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with said antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of said fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit 3-Isopropylmalate dehydratase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. 3-Isopropylmalate dehydratase catalyzes the irreversible or reversible reaction 2-Isopropylmalate and H_2O = 3-Isopropylmalate (see Figure 1). Methods for detection of 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 2-Isopropylmalate and H_2O with a 3-Isopropylmalate dehydratase;

b) contacting 2-Isopropylmalate and H_2O with 3-Isopropylmalate dehydratase and said test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 3-Isopropylmalate with a 3-Isopropylmalate dehydratase;

b) contacting 3-Isopropylmalate with a 3-Isopropylmalate dehydratase and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

Enzymatically active fragments of a fungal 3-Isopropylmalate dehydratase are also useful in the methods of the invention. For example, an enzymatically active polypeptide comprising at least 100 consecutive amino acid residues of a fungal 3-Isopropylmalate dehydratase may be used in the methods of the invention. In addition, an enzymatically active polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95% or at least 98% sequence identity with a fungal 3-Isopropylmalate dehydratase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal 3-Isopropylmalate dehydratase and at least 10%, 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 2-Isopropylmalate and H_2O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase and having at least 10% of the activity

thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 3-Isopropylmalate dehydratase;

b) contacting 2-Isopropylmalate and H_2O with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate;

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 3-Isopropylmalate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase and at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 3-Isopropylmalate dehydratase;

b) contacting 3-Isopropylmalate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following, 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate;

wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

For the *in vitro* enzymatic assays, 3-Isopropylmalate dehydratase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of 3-Isopropylmalate dehydratase may be described in (Bigelis and Umbarger (1975) J Biol Chem *250*: 4315 – 21 (PMID: 1126953); Kohlhaw (1988) Meth Enzymol *166*: 423 – 9 (PMID: 3071717)). Other

methods for the purification of 3-Isopropylmalate dehydratase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a 3-Isopropylmalate dehydratase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said 3-Isopropylmalate dehydratase in said cell, cells, tissue, or organism; and

c) comparing the expression of 3-Isopropylmalate dehydratase in steps (a) and (b);

wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

Expression of 3-Isopropylmalate dehydratase can be measured by detecting the IPMD1 primary transcript or mRNA, 3-Isopropylmalate dehydratase polypeptide, or 3-Isopropylmalate dehydratase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology* Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for detecting IPMD1 RNA include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using an IPMD1 promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect IPMD1 protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with IPMD1, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators, preferably inhibitors, of IPMD1 expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided is a method of screening for an antibiotic by determining whether a test compound is active against the gene identified (SEQ ID NO: 10 or SEQ ID NO: 11), its gene product (SEQ ID NO: 12), or the biochemical pathway or pathways it functions on.

In one particular embodiment, the method is performed by providing an organism having a first form of the gene corresponding to either SEQ ID NO: 10 or SEQ ID NO: 11, either a normal form, a mutant form, a homologue, or a heterologous IPMD1 gene that performs a similar function as IPMD1. The first form of IPMD1 may or may not confer a growth conditional phenotype, *i.e.*, a L-leucine requiring phenotype, and/or a hypersensitivity or hyposensitivity phenotype on the organism having that altered form. In one particular embodiment a mutant form contains a transposon insertion. A comparison organism having a second form of an IPMD1, different from the first form of the gene is also provided, and the two organisms are separately contacted with a test compound. The growth of the two organisms in the presence of the test compound is then compared.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a 3-Isopropylmalate dehydratase gene, and providing comparison cells having a different form of a 3-Isopropylmalate dehydratase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and said comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that the optional determination of the growth of said first organism and said comparison second organism in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different genes. It is also recognized that any combination of two different forms of an

IPMD1 gene, including normal genes, mutant genes, homologues, and functional homologues may be used in this method. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which IPMD1 functions, comprising:

j) providing cells having one form of a gene in the L-leucine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;

k) contacting said cells and said comparison cells with a test compound; and

l) determining the growth of said cells and said comparison cells in the presence of said test compound;

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent

increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which IPMD1 functions, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-leucine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism;

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that determination of the growth of said organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

The present inventors have discovered that disruption of the THR4 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that Threonine synthase is a target for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit THR4 gene expression or biological activity of its gene product(s). Such methods include ligand binding assays, assays for enzyme activity, cell-based assays, and assays for THR4 gene expression. Any compound that is a ligand for Threonine synthase may have antibiotic activity. For the purposes of the invention, "ligand" refers to a molecule that will bind to a site on a polypeptide. The compounds identified by the methods of the invention are useful as antibiotics.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a Threonine synthase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said Threonine synthase polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

The Threonine synthase protein may have the amino acid sequence of a naturally occurring Threonine synthase found in a fungus, animal, plant, or microorganism, or may have an amino acid sequence derived from a naturally occurring sequence. Preferably the Threonine synthase is a fungal Threonine synthase. The cDNA (SEQ ID NO: 13) encoding the Threonine synthase protein, the genomic DNA (SEQ ID NO: 14) encoding the *M. grisea* protein, and the polypeptide (SEQ ID NO: 15) can be found herein.

In one aspect, the invention also provides for a polypeptide consisting essentially of SEQ ID NO: 15. For the purposes of the invention, a polypeptide consisting essentially of SEQ ID NO: 15 has at least 80% sequence identity with SEQ ID NO: 15 and catalyses the interconversion of O-phospho-L-homoserine and water with L-threonine and orthophosphate with at least 10% of the activity of SEQ ID NO: 15. Preferably, the polypeptide consisting essentially of SEQ ID NO: 15 has at least 85% sequence identity with SEQ ID NO: 15, more preferably the sequence identity is at least 90%, most preferably the sequence identity is at least 95% or 97 or 99%, or any integer from 80-100% sequence identity in ascending order. And, preferably, the polypeptide consisting essentially of SEQ ID NO: 15 has at least 25%, at least 50%, at least 75% or at

least 90% of the activity of *M. grisea* Threonine synthase, or any integer from 60-100% activity in ascending order.

By "fungal Threonine synthase" is meant an enzyme that can be found in at least one fungus, and which catalyzes the interconversion of O-phospho-L-homoserine and water with L-threonine and orthophosphate. The Threonine synthase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

In one embodiment, the Threonine synthase is a *Magnaporthe* Threonine synthase. *Magnaporthe* species include, but are not limited to, *Magnaporthe rhizophila, Magnaporthe salvinii, Magnaporthe grisea* and *Magnaporthe poae* and the imperfect states of *Magnaporthe* in the genus *Pyricularia*. Preferably, the *Magnaporthe* Threonine synthase is from *Magnaporthe grisea*.

In various embodiments, the Threonine synthase can be from Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus (*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), and the like.

Fragments of a Threonine synthase polypeptide may be used in the methods of the invention, preferably if the fragments include an intact or nearly intact epitope that occurs on the biologically active wildtype Threonine synthase. The fragments comprise at least

10 consecutive amino acids of a Threonine synthase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, or at least 540 consecutive amino acids residues of a Threonine synthase. In one embodiment, the fragment is from a *Magnaporthe* Threonine synthase. Preferably, the fragment contains an amino acid sequence conserved among fungal Threonine synthases.

Polypeptides having at least 50% sequence identity with a fungal Threonine synthase are also useful in the methods of the invention. Preferably, the sequence identity is at least 60%, more preferably the sequence identity is at least 70%, most preferably the sequence identity is at least 80% or 90 or 95 or 99%, or any integer from 60-100% sequence identity in ascending order.

In addition, it is preferred that the polypeptide has at least 10% of the activity of a fungal Threonine synthase. More preferably, the polypeptide has at least 25%, at least 50%, at least 75% or at least 90% of the activity of a fungal Threonine synthase. Most preferably, the polypeptide has at least 10%, at least 25%, at least 50%, at least 75% or at least 90% of the activity of the *M. grisea* Threonine synthase protein.

Thus, in another embodiment, the invention provides a method for identifying a test compound as a candidate for a fungicide, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal Threonine synthase; a polypeptide having at least 50% sequence identity with a fungal Threonine synthase; and a polypeptide having at least 10% of the activity of a fungal Threonine synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

Any technique for detecting the binding of a ligand to its target may be used in the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and

include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with a Threonine synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound Threonine synthase is detected. In a preferred embodiment, bound Threonine synthase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay, Threonine synthase is labeled prior to contacting the immobilized candidate ligands. Preferred labels include fluorescent or radioactive moieties. Preferred detection methods include fluorescence correlation spectroscopy (FCS) and FCS-related confocal nanofluorimetric methods.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit Threonine synthase enzymatic activity. The compounds can be tested using either *in vitro* or cell based assays. Alternatively, a compound can be tested by applying it directly to a fungus or fungal cell, or expressing it therein, and monitoring the fungus or fungal cell for changes or decreases in growth, development, viability, pathogenicity, or alterations in gene expression. Thus, in one embodiment, the invention provides a method for determining whether a compound identified as an antibiotic candidate by an above method has antifungal activity, further comprising: contacting a fungus or fungal cells with said antifungal candidate and detecting a decrease in the growth, viability, or pathogenicity of said fungus or fungal cells.

By decrease in growth, is meant that the antifungal candidate causes at least a 10% decrease in the growth of the fungus or fungal cells, as compared to the growth of the fungus or fungal cells in the absence of the antifungal candidate. By a decrease in viability is meant that at least 20% of the fungal cells, or portion of the fungus contacted with the antifungal candidate are nonviable. Preferably, the growth or viability will be decreased by at least 40%. More preferably, the growth or viability will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal growth and cell viability are known to those skilled in the art. By decrease in pathogenicity, is meant that the antifungal candidate causes at least a 10% decrease in the disease caused by contact of the fungal pathogen with its host, as compared to the disease caused in the

absence of the antifungal candidate. Preferably, the disease will be decreased by at least 40%. More preferably, the disease will be decreased by at least 50%, 75% or at least 90% or more. Methods for measuring fungal disease are well known to those skilled in the art, and include such metrics as lesion formation, lesion size, sporulation, respiratory failure, and/or death.

The ability of a compound to inhibit Threonine synthase activity can be detected using *in vitro* enzymatic assays in which the disappearance of a substrate or the appearance of a product is directly or indirectly detected. Threonine synthase catalyzes the irreversible or reversible reaction O-phospho-L-homoserine and water = L-threonine and orthophosphate (see Figure 1). Methods for detection of O-phospho-L-homoserine, L-threonine, orthophosphate, and water, include spectrophotometry, mass spectroscopy, thin layer chromatography (TLC) and reverse phase HPLC.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting O-phospho-L-homoserine and water with a Threonine synthase;

b) contacting O-phospho-L-homoserine and water with Threonine synthase and said test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-threonine and orthophosphate with a Threonine synthase;

b) contacting L-threonine and orthophosphate with a Threonine synthase and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

Enzymatically active fragments of a fungal Threonine synthase are also useful in the methods of the invention. For example, an enzymatically active polypeptide comprising at least 100 consecutive amino acid residues of a fungal Threonine synthase may be used in the methods of the invention. In addition, an enzymatically active polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95% or at least 98% sequence identity with a fungal Threonine synthase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal Threonine synthase and at least 10%, 25%, 75% or at least 90% of the activity thereof.

Thus, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting O-phospho-L-homoserine and water with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a Threonine synthase, and a polypeptide having at least 50% sequence identity with a Threonine synthase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a Threonine synthase;

b) contacting O-phospho-L-homoserine and water with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

An additional method is provided by the invention for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-threonine and orthophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a Threonine synthase, and a polypeptide having at least 50% sequence identity with a Threonine synthase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a Threonine synthase;

b) contacting L-threonine and orthophosphate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following, O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances indicates that said test compound is a candidate for an antibiotic.

For the *in vitro* enzymatic assays, Threonine synthase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archael, bacterial, fungal, or other eukaryotic cell culture. Preferably these proteins are produced using an *E. coli*, yeast, or filamentous fungal expression system. Methods for the purification of Threonine synthase may be described in Malumbres *et al.* (1994) Appl Environ Microbiol *60*: 2209 – 19 (PMID: 8074505). Other methods for the purification of Threonine synthase proteins and polypeptides are known to those skilled in the art.

As an alternative to *in vitro* assays, the invention also provides cell based assays. In one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a Threonine synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said Threonine synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of Threonine synthase in steps (a) and (b), wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

Expression of Threonine synthase can be measured by detecting the THR4 primary transcript or mRNA, Threonine synthase polypeptide, or Threonine synthase enzymatic activity. Methods for detecting the expression of RNA and proteins are known to those skilled in the art. See, for example, *Current Protocols in Molecular Biology*

Ausubel *et al.*, eds., Greene Publishing and Wiley-Interscience, New York, 1995. The method of detection is not critical to the invention. Methods for detecting THR4 RNA include, but are not limited to amplification assays such as quantitative reverse transcriptase-PCR, and/or hybridization assays such as Northern analysis, dot blots, slot blots, in-situ hybridization, transcriptional fusions using a THR4 promoter fused to a reporter gene, DNA assays, and microarray assays.

Methods for detecting protein expression include, but are not limited to, immunodetection methods such as Western blots, ELISA assays, polyacrylamide gel electrophoresis, mass spectroscopy, and enzymatic assays. Also, any reporter gene system may be used to detect THR4 protein expression. For detection using gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame with THR4, so as to produce a chimeric polypeptide. Methods for using reporter systems are known to those skilled in the art.

Chemicals, compounds or compositions identified by the above methods as modulators, preferably inhibitors, of THR4 expression or activity can then be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

Antifungals and antifungal inhibitor candidates identified by the methods of the invention can be used to control the growth of undesired fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

Examples of undesired fungi include, but are not limited to Powdery Scab (*Spongospora subterranea*), Grey Mould (*Botrytis cinerea*), White Rot (*Armillaria mellea*), Heartrot Fungus (*Ganoderma adspersum*), Brown-Rot (*Piptoporus betulinus*), Corn Smut (*Ustilago maydis*), Heartrot (*Polyporus squamosus*), Gray Leaf Spot (*Cercospora zeae-maydis*), Honey Fungus (*Armillaria gallica*), Root rot (*Armillaria luteobubalina*), Shoestring Rot (*Armillaria ostoyae*), Banana Anthracnose Fungus

(*Colletotrichum musae*), Apple-rotting Fungus (*Monilinia fructigena*), Apple-rotting Fungus (*Penicillium expansum*), Clubroot Disease (*Plasmodiophora brassicae*), Potato Blight (*Phytophthora infestans*), Root pathogen (*Heterobasidion annosum*), Take-all Fungus (*Gaeumannomyces graminis*), Dutch Elm Disease (*Ophiostoma ulmi*), Bean Rust (*Uromyces appendiculatus*), Northern Leaf Spot (*Cochliobolus carbonum*), Milo Disease (*Periconia circinata*), Southern Corn Blight (*Cochliobolus heterostrophus*), Leaf Spot (*Cochliobolus lunata*), Brown Stripe (*Cochliobolus stenospilus*), Panama disease (*Fusarium oxysporum*), Wheat Head Scab Fungus (*Fusarium graminearum*), Cereal Foot Rot (*Fusarium culmorum*), Potato Black Scurf (*Rhizoctonia solani*), Wheat Black Stem Rust (*Puccinia graminis*), White mold (*Sclerotinia sclerotiorum*), diseases of animals such as infections of lungs, blood, brain, skin, scalp, nails or other tissues (*Aspergillus fumigatus Aspergillus* sp. *Fusraium* sp., *Trichophyton* sp., *Epidermophyton* sp., and *Microsporum* sp., and the like).

Also provided is a method of screening for an antibiotic by determining whether a test compound is active against the gene identified (SEQ ID NO: 13 or SEQ ID NO: 14), its gene product (SEQ ID NO: 15), or the biochemical pathway or pathways on which it functions.

In one particular embodiment, the method is performed by providing an organism having a first form of the gene corresponding to either SEQ ID NO: 13 or SEQ ID NO: 14, either a normal form, a mutant form, a homologue, or a heterologous THR4 gene that performs a similar function as THR4. The first form of THR4 may or may not confer a growth conditional phenotype, *i.e.*, a L-threonine requiring phenotype, and/or a hypersensitivity or hyposensitivity phenotype on the organism having that altered form. In one particular embodiment a mutant form contains a transposon insertion. A comparison organism having a second form of a THR4, different from the first form of the gene is also provided, and the two organisms are separately contacted with a test compound. The growth of the two organisms in the presence of the test compound is then compared.

Thus, in one embodiment, the invention provides a method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a Threonine synthase gene, and providing comparison cells having a different form of a Threonine synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and said comparison cells in the presence of the test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that the optional determination of the growth of said first organism and said comparison second organism in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different genes. It is also recognized that any combination of two different forms of a THR4 gene, including normal genes, mutant genes, homologues, and functional homologues may be used in this method. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment the organism is *Magnaporthe grisea*.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry, New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which THR4 functions, comprising:

m) providing cells having one form of a gene in the L-threonine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;

n) contacting said cells and said comparison cells with a test compound; and

o) determining the growth of said cells and said comparison cells in the presence of said test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger *et al.* (1993) Principles of Biochemistry).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which THR4 functions, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-threonine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism, wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

It is recognized in the art that determination of the growth of said organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

EXPERIMENTAL

Example 1

Construction of Plasmids with a Transposon Containing a Selectable Marker.

Construction of Sif transposon: Sif was constructed using the GPS3 vector from the GPS-M mutagenesis system from New England Biolabs, Inc. (Beverly, MA) as a backbone. This system is based on the bacterial transposon Tn7. The following manipulations were done to GPS3 according to Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press. The kanamycin resistance gene (npt) contained between the Tn7 arms was removed by EcoRV digestion. The bacterial hygromycin B phosphotransferase (hph) gene (Gritz and Davies (1983) Gene *25*: 179 – 88 (PMID: 6319235)) under control of the *Aspergillus nidulans* trpC promoter and terminator (Mullaney *et al.* (1985) Mol Gen Genet *199*: 37 – 45 (PMID: 3158796)) was cloned by a HpaI/EcoRV blunt ligation into the Tn7 arms of the GPS3 vector yielding pSif1. Excision of the ampicillin resistance gene (bla) from pSif1 was achieved by cutting pSif1 with XmnI and BglI followed by a T4 DNA polymerase treatment to remove the 3' overhangs left by the BglI digestion and religation of the plasmid to yield pSif. Top 10F' electrocompetent *E. coli* cells (Invitrogen) were transformed with ligation mixture according to manufacturer's recommendations. Transformants containing the Sif transposon were selected on LB agar (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual) containing 50ug/ml of hygromycin B (Sigma Chem. Co., St. Louis, MO).

Example 2

Construction of a Fungal Cosmid Library

Cosmid libraries were constructed in the pcosKA5 vector (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)) as described in Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual. Cosmid libraries were quality checked by pulsed-field gel electrophoresis, restriction digestion analysis, and PCR identification of single genes.

Example 3

Construction of Cosmids with Transposon Insertion into Fungal Genes

Sif Transposition into a Cosmid: Transposition of Sif into the cosmid framework was carried out as described by the GPS-M mutagenesis system (New England Biolabs, Inc.). Briefly, 2ul of the 10X GPS buffer, 70 ng of supercoiled pSIF, 8-12 ug of target cosmid DNA were mixed and taken to a final volume of 20ul with water. 1ul of transposase (TnsABC) was added to the assembly reaction and incubated for 10 minutes at 37°C. After the assembly reaction, 1ul of start solution was added to the tube, mixed well and incubated for 1 hour at 37°C followed by heat inactivation of the proteins at 75°C for 10 min. Destruction of the remaining untransposed pSif was done by PISceI digestion at 37°C for 2 hours followed by 10 min incubation at 75°C to inactivate the proteins. Transformation of Top10F' electrocompetent cells (Invitrogen) was done according to manufacturers recommendations. Sif-containing cosmid transformants were selected by growth on LB agar plates containing 50ug/ml of hygromycin B (Sigma Chem. Co.) and 100 ug/ml of Ampicillin (Sigma Chem. Co.).

Example 4

High Throughput Preparation and Verification of Transposon Insertion into the M. grisea ASN1 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory

Press) supplemented with 50 ug/ml of ampicillin. Blocks were incubated with shaking at 37°C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.* (1997) Genome Res *7*: 1072 – 84 (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* ASN1 gene was chosen for further analysis. This construct was designated cpgmra0011008a10 and it contains the SIF transposon approximately between amino acids 345 and 346 relative to the *Saccharomyces cerevisiae* homologue (total length: 572 amino acids, GENBANK: 6325403).

Example 5

Preparation of ASN1 Cosmid DNA and Transformation of Magnaporthe grisea

Cosmid DNA from the ASN1 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (QIAGEN), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.* (1997) MPMI *10*: 700 - 708). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) shaking at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of $2x10^8$ protoplasts/ml. 50ul protoplast suspension was mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose for one day, then overlayed with CM agar media containing hygromycin B (250ug/ml) to

select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)). Two independent strains were identified and are hereby referred to as KO1-2 and KO1-8, respectively.

Example 6

Effect of Transposon Insertion on Magnaporthe pathogenicity

The target fungal strains, KO1-2 and KO1-8, obtained in Example 5 and the wild type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-week period. Rice infection assays were performed using Indian rice cultivar CO39 essentially as described in Valent *et al.* ((1991) Genetics *127*: 87 - 101 (PMID: 2016048)). All three strains were grown for spore production on complete agar media. Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of conidial suspension (5 x 10^4 conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of ASN1 gene disruption on *Magnaporthe* infection at five days post-inoculation.

Example 7

Verification of ASN1 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-2 and KO1-8, containing the ASN1 disrupted gene obtained in Example 5 were analyzed for their nutritional requirement for L-asparagine using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The innoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$, 6.7mM KCl, 3.5mM Na_2SO_4, 11mM KH_2PO_4, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM $MgCl_2$, 1.0mM $CaCl_2$ and trace elements, pH adjusted to 6.0 with NaOH. Final

concentrations of trace elements are: 7.6μM $ZnCl_2$, 2.5μM $MnCl_2 \cdot 4H_2O$, 1.8μM $FeCl_2 \cdot 4H_2O$, 0.71μM $CoCl_2 \cdot 6H_2O$, 0.64μM $CuCl_2 \cdot 2H_2O$, 0.62μM Na_2MoO_4, 18μM H_3BO_3. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to $2x10^5$ spores/ml. 100μl of spore suspension were deposited into each well of the microtiter plates. The plates were incubated at 25°C for 7 days. Optical density (OD) measurements at 490nm and 750nm were taken daily. The OD_{490} measures the extent of tetrazolium dye reduction and the level of growth, and OD_{750} measures growth only. Turbidity = $OD_{490} + OD_{750}$. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of L-asparagine.

Example 8

Cloning and Expression Strategies, Extraction and Purification of Asparagine Synthase Protein.

The following protocol may be employed to obtain a purified Asparagine Synthase protein.

Cloning and expression strategies:

An ASN1 cDNA gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein can be evaluated by SDS-PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer by sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 9

Assays for Testing Binding of Test Compounds to Asparagine Synthase

The following protocol may be employed to identify test compounds that bind to the Asparagine Synthase protein.

- Purified full-length Asparagine Synthase polypeptide with a His/fusion protein tag (Example 8) is bound to a HisGrab™ Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, as may be described in Luehr and Schuster (1980) J Biochem Biophys Methods *3*: 151 - 61 (PMID: 6108975)) for binding of radiolabeled L-[4-^{14}C]aspartate (Dearing and Walker (1960) Nature *185*: 690 - 691) to the bound Asparagine Synthase.
- Screening of test compounds is performed by adding test compound and L-[4-^{14}C]aspartate (Dearing and Walker (1960) Nature *185*: 690 - 691) to the wells of the HisGrab™ plate containing bound Asparagine Synthase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (Scintiverse®, Fisher Scientific) is added to each well.
- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* Asparagine Synthase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the ASN1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 8). Oligonucleotide primers are designed to amplify a portion of the ASN1 gene using the polymerase chain

reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 8 above.

Test compounds that bind ASN1 are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 10

Assays for Testing Inhibitors or Candidates for Inhibition of Asparagine Synthase Activity

The enzymatic activity of Asparagine Synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Luehr and Schuster (1980) J Biochem Biophys Methods *3*: 151 - 61 (PMID: 6108975). Candidate compounds are identified when a decrease in products or a lack of decrease in substrates is detected with the reaction proceeding in either direction.

Additionally, the enzymatic activity of a polypeptide comprising 10-50 amino acids from the *M. grisea* Asparagine Synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Luehr and Schuster (1980) J Biochem Biophys Methods *3*: 151 - 61 (PMID: 6108975). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the ASN1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 8). Oligonucleotide primers are designed to amplify a portion of the ASN1 gene using polymerase chain reaction amplification method. The DNA fragment

encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed and purified as described in Example 8 above.

Test compounds identified as inhibitors of ASN1 activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2×10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 11

Assays for Testing Compounds for Alteration of Asparagine Synthase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem®, La Jolla, CA), washed with water and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL® Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.* (1989) Molecular

Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the ASN1 gene as a probe. Test compounds resulting in a reduced level of ASN1 mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Example 12

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Asparagine Synthase with No Activity

Magnaporthe grisea fungal cells containing a mutant form of the ASN1 gene which abolishes enzyme activity, such as a gene containing a transposon insertion (see Examples 4 and 5), are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-asparagine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-asparagine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 13

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Asparagine Synthase with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of the ASN1 gene, such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-asparagine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 14

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-asparagine Biosynthetic Gene with No Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-asparagine biosynthetic pathway (*e.g.* Formiminoaspartate deiminase (E.C. 3.5.3.5)) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-asparagine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-asparagine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 15

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-asparagine Biosynthetic Gene with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-asparagine biosynthetic pathway (*e.g.* Formiminoaspartate deiminase (E.C. 3.5.3.5)), such as a promoter truncation that reduces expression, are grown under standard fungal

growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-asparagine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 16

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Fungal ASN1 and a Second Fungal Strain Containing a Heterologous ASN1 Gene

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional ASN1 gene and containing an asparagine synthetase B gene from *Vibrio cholerae* (Genbank 11272666, 50% sequence identity) are grown under standard fungal

growth conditions that are well known and described in the art. A *M. grisea* strain carrying a heterologous ASN1 gene is made as follows:

- A *M. grisea* strain is made with a nonfunctional ASN1 gene, such as one containing a transposon insertion in the native gene (see Examples 4 and 5).
- A construct containing a heterologous ASN1 gene is made by cloning the asparagine synthetase B gene from *Vibrio cholerae* into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll *et al.* (1994) Fungal Gen News Lett *41*: 22) using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual).
- The said construct is used to transform the *M. grisea* strain lacking a functional ASN1 gene (see Example 5). Transformants are selected on minimal agar medium lacking L-asparagine. Only transformants carrying a functional ASN1 gene will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of ASN1 are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with

specificity to the native or heterologous ASN1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 17

Pathway Specific In Vivo Assay Screening Protocol

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing 4 mM L-asparagine (Sigma-Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 7). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing 4 mM L-asparagine. Test compounds are added to wells containing spores in minimal media and minimal media containing L-asparagine. The total volume in each well is 200µl. Both minimal media and L-asparagine containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the L-asparagine biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well containing L-asparagine as a result of the addition of the test compound. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 18

High Throughput Preparation and Verification of Transposon Insertion into the M. grisea ALAS1 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) supplemented with 50 ug/ml of ampicillin. Blocks were incubated with shaking at 37 C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.* (1997) Genome Res *7*: 1072 – 84 (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* ALAS1 gene was chosen for further analysis. This construct was designated cpgmra0011005e01 and it contains the SIF transposon at approximately amino acid 100 relative to the *Aspergillus nidulans* homologue *HEMA* (total length: 648 amino acids, GENBANK: 585244 (SWISS-PROT: P38092)).

Example 19

Preparation of ALAS1 Cosmid DNA and Transformation of Magnaporthe grisea

Cosmid DNA from the ALAS1 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (QIAGEN), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.* (1997) MPMI *10*: 700 - 708). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) shaking at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of $2x10^8$ protoplasts/ml. 50ul protoplast suspension was

mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose and 200 μM 5-aminolevulinic acid (Sigma-Aldritch Co.) for one day, then overlayed with CM agar media containing hygromycin B (250ug/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)). Two independent strains were identified and are hereby referred to as KO1-1 and KO1-106, respectively.

Example 20

Effect of Transposon Insertion on Magnaporthe pathogenicity

The target fungal strains, KO1-1 and KO1-106, obtained in Example 19 and the wild type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-week period. Rice infection assays were performed using Indian rice cultivar CO39 essentially as described in Valent *et al.* ((1991) Genetics *127*: 87 - 101 (PMID: 2016048)). All three strains were grown for spore production on complete agar media. Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of conidial suspension (5 x 10^4 conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of ALAS1 gene disruption on *Magnaporthe* infection at five days post-inoculation.

Example 21

Verification of ALAS1 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-1 and KO1-106, containing the ALAS1 disrupted gene obtained in Example 19 were analyzed for their nutritional requirement for 5-aminolevulinic acid using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The innoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$, 6.7mM KCl, 3.5mM Na_2SO_4, 11mM KH_2PO_4, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM $MgCl_2$, 1.0mM $CaCl_2$ and trace elements. Final concentrations of trace elements are: 7.6μM $ZnCl_2$, 2.5μM $MnCl_2 \cdot 4H_2O$, 1.8μM $FeCl_2 \cdot 4H_2O$, 0.71μM $CoCl_2 \cdot 6H_2O$, 0.64μM $CuCl_2 \cdot 2H_2O$, 0.62μM Na_2MoO_4, 18μM H_3BO_3. pH adjusted to 6.0 with NaOH. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to $2x10^5$ spores/ml. 100μl of spore suspension were deposited into each well of the microtiter plates. The plates were incubated at 25°C for 7 days. Optical density (OD) measurements at 490nm and 750nm were taken daily. The OD_{490} measures the extent of tetrazolium dye reduction and the level of growth, and OD_{750} measures growth only. Turbidity = $OD_{490} + OD_{750}$. Data confirming the annotated gene function is presented in Figure 3, and as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of 5-aminolevulinate.

Example 22

Cloning and Expression Strategies, Extraction and Purification of 5-Aminolevulinate Synthase Protein.

The following protocol may be employed to obtain a purified 5-Aminolevulinate synthase protein.

Cloning and expression strategies:

An ALAS1 cDNA gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein can be evaluated by SDS-PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer by sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 23

Assays for Testing Binding of Test Compounds to 5-Aminolevulinate Synthase

The following protocol may be employed to identify test compounds that bind to the 5-Aminolevulinate synthase protein.

- Purified full-length 5-Aminolevulinate synthase polypeptide with a His/fusion protein tag (Example 22) is bound to a HisGrab™ Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, Shoolingin-Jordan *et al.* (1997) Methods Enzymol *281*: 309 - 16 (PMID: 9250995)) for binding of radiolabeled succinyl-CoA (custom made, PerkinElmer Life Sciences, Inc., Boston, MA) to the bound 5-Aminolevulinate synthase.
- Screening of test compounds is performed by adding test compound and radiolabeled succinyl-CoA (custom made, PerkinElmer Life Sciences, Inc., Boston, MA) to the wells of the HisGrab™ plate containing bound 5-Aminolevulinate synthase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (Scintiverse®, Fisher Scientific) is added to each well.

- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* 5-Aminolevulinate synthase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the ALAS1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 22). Oligonucleotide primers are designed to amplify a portion of the ALAS1 gene using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 22 above.

Test compounds that bind ALAS1 are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 24

Assays for Testing Inhibitors or Candidates for Inhibition of 5-Aminolevulinate Synthase Activity

The enzymatic activity of 5-Aminolevulinate synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Shoolingin-Jordan *et al.* (1997) Methods Enzymol *281*: 309 - 16 (PMID: 9250995). Candidate compounds are identified when a decrease in products or a lack of decrease in substrates is detected with the reaction proceeding in either direction.

Additionally, the enzymatic activity of a polypeptide comprising 10-50 amino acids from the *M. grisea* 5-Aminolevulinate synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Shoolingin-Jordan *et al.* (1997) Methods Enzymol *281*: 309 - 16 (PMID: 9250995). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the ALAS1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 22). Oligonucleotide primers are designed to amplify a portion of the ALAS1 gene using polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed and purified as described in Example 22 above.

Test compounds identified as inhibitors of ALAS1 activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2×10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 25

Assays for Testing Compounds for Alteration of 5-Aminolevulinate Synthase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a

minimal growth medium to a concentration of $2x10^5$ spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem®, La Jolla, CA), washed with water and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL® Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the ALAS1 gene as a probe. Test compounds resulting in a reduced level of ALAS1 mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Example 26

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of 5-Aminolevulinate Synthase with No Activity

Magnaporthe grisea fungal cells containing a mutant form of the ALAS1 gene which abolishes enzyme activity, such as a gene containing a transposon insertion (see Examples 18 and 19), are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 200 μM 5-aminolevulinate (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 20 μM 5-aminolevulinate to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each

compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 27

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of 5-Aminolevulinate Synthase with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of the ALAS1 gene, such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 200 µM 5-aminolevulinate (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is

calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 28

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a Heme Biosynthetic Gene with No Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the heme biosynthetic pathway (*e.g.* Aminolevulinate dehydratase (E.C. 4.2.1.24)) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 200 μM 5-aminolevulinate (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 20 μM 5-aminolevulinate to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 29

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a Heme Biosynthetic Gene with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the heme biosynthetic pathway (*e.g.* Aminolevulinate dehydratase (E.C. 4.2.1.24)), such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 200 μM 5-aminolevulinate (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 30

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing M. grisea ALAS1 and a Second Fungal Strain Containing a Heterologous ALAS1 Gene

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional ALAS1 gene and containing a 5-Aminolevulinic Acid Synthase gene from *Candida albicans* (Genbank: 10720014, SWISS-PROT: O94069, 54% sequence identity) are grown under standard fungal growth conditions that are well known and described in the art. A *M. grisea* strain carrying a heterologous ALAS1 gene is made as follows:

- A *M. grisea* strain is made with a nonfunctional ALAS1 gene, such as one containing a transposon insertion in the native gene (see Examples 18 and 19).
- A construct containing a heterologous ALAS1 gene is made by cloning the 5-Aminolevulinic Acid Synthase gene from *Candida albicans* into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll *et al.* (1994) Fungal Gen News Lett *41*: 22) using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press).
- The said construct is used to transform the *M. grisea* strain lacking a functional ALAS1 gene (see Example 19). Transformants are selected on minimal agar medium lacking 5-aminolevulinate. Only transformants carrying a functional ALAS1 gene will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of ALAS1 are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are

incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous ALAS1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 31

Pathway Specific In Vivo Assay Screening Protocol

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing 200 μM 5-aminolevulinate (Sigma-Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 21). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing 200 μM 5-aminolevulinate. Test compounds are added to wells containing spores in minimal media and minimal media containing 5-aminolevulinate. The total volume in each well is 200μl. Both minimal media and 5-aminolevulinate containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the heme biosynthetic pathway when the observed growth in the

well containing minimal media is less than the observed growth in the well containing 5-aminolevulinate as a result of the addition of the test compound. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 32

High Throughput Preparation and Verification of Transposon Insertion into the M. grisea HISP1 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) supplemented with 50 ug/ml of ampicillin. Blocks were incubated with shaking at 37 C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.* (1997) Genome Res *7*: 1072 – 84 (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* HISP1 gene was chosen for further analysis. This construct was designated cpgmra0012021b05 and it contains the SIF transposon approximately 100 nucleotides before the start codon relative to the *Schizosaccharomyces pombe* homologue (total length: 315 amino acids, GENBANK: 3183028, SWISS-PROT: O14059), and is predicted to eliminate or reduce gene function.

Example 33

Preparation of HISP1 Cosmid DNA and Transformation of Magnaporthe grisea

Cosmid DNA from the HISP1 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (QIAGEN), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.* (1997) MPMI *10*: 700 - 708). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) shaking at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of $2x10^8$ protoplasts/ml. 50ul protoplast suspension was mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose for one day, then overlayed with CM agar media containing hygromycin B (250ug/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)). Two independent strains were identified and are hereby referred to as KO1-1 and KO1-3, respectively.

Example 34

Effect of Transposon Insertion on Magnaporthe pathogenicity

The target fungal strains, KO1-1 and KO1-3, obtained in Example 33 and the wild type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-week period. Rice infection assays were performed using Indian rice cultivar CO39 essentially as described in Valent *et al.* ((1991) Genetics *127*: 87 - 101 (PMID: 2016048)). All three strains were grown for spore production on complete agar media. Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of conidial suspension (5 x 10^4 conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan

monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of HISP1 gene disruption on *Magnaporthe* infection at five days post-inoculation.

Example 35

Verification of HISP1 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-1 and KO1-3, containing the HISP1 disrupted gene obtained in Example 33 were analyzed for their nutritional requirement for histidine using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The innoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$, 6.7mM KCl, 3.5mM Na_2SO_4, 11mM KH_2PO_4, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM $MgCl_2$, 1.0mM $CaCl_2$ and trace elements. Final concentrations of trace elements are: 7.6μM $ZnCl_2$, 2.5μM $MnCl_2 \cdot 4H_2O$, 1.8μM $FeCl_2 \cdot 4H_2O$, 0.71μM $CoCl_2 \cdot 6H_2O$, 0.64μM $CuCl_2 \cdot 2H_2O$, 0.62μM Na_2MoO_4, 18μM H_3BO_3. pH adjusted to 6.0 with NaOH. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to $2x10^5$ spores/ml. 100μl of spore suspension were deposited into each well of the microtiter plates. The plates were incubated at 25°C for 7 days. Optical density (OD) measurements at 490nm and 750nm were taken daily. The OD_{490} measures the extent of tetrazolium dye reduction and the level of growth, and OD_{750} measures growth only. Turbidity = $OD_{490} + OD_{750}$. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of L-histidine.

Example 36

Cloning and Expression Strategies, Extraction and Purification of Histidinol-phosphatase Protein.

The following protocol may be employed to obtain a purified histidinol-phosphatase protein.

Cloning and expression strategies:

A HISP1 cDNA gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags and the expression of recombinant protein can be evaluated by SDS-PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer by sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 37

Assays for Testing Binding of Test Compounds to Histidinol-phosphatase

The following protocol may be employed to identify test compounds that bind to the histidinol-phosphatase protein.

- Purified full-length histidinol-phosphatase polypeptide with a His/fusion protein tag (Example 36) is bound to a HisGrab™ Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, Millay and Houston (1973) Biochemistry *12*: 2591 – 2596 (PMID: 4351203)) for binding of radiolabeled L-Histidinol phosphate (custom made, PerkinElmer Life Sciences, Inc., Boston, MA) to the bound histidinol-phosphatase.
- Screening of test compounds is performed by adding test compound and L-Histidinol phosphate (custom made, PerkinElmer Life Sciences, Inc., Boston, MA) to the wells of the HisGrab™ plate containing bound histidinol-phosphatase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (Scintiverse®, Fisher Scientific) is added to each well.
- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* histidinol-phosphatase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the HISP1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 36). Oligonucleotide primers are designed to amplify a portion of the HISP1 gene using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 36 above.

Test compounds that bind HISP1 are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density

measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 38

Assays for Testing Inhibitors or Candidates for Inhibition of Histidinol-phosphatase Activity

The enzymatic activity of histidinol-phosphatase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Millay and Houston (1973) Biochemistry *12*: 2591 – 2596 (PMID: 4351203). Candidate compounds are identified when a decrease in products or a lack of decrease in substrates is detected with the reaction proceeding in either direction.

Additionally, the enzymatic activity of a polypeptide comprising 10-50 amino acids from the *M. grisea* histidinol-phosphatase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Millay and Houston (1973) Biochemistry *12*: 2591 – 2596 (PMID: 4351203). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the HISP1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 36). Oligonucleotide primers are designed to amplify a portion of the HISP1 gene using polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed and purified as described in Example 36 above.

Test compounds identified as inhibitors of HISP1 activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2×10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates

are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 39

Assays for Testing Compounds for Alteration of Histidinol-phosphatase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem®, La Jolla, CA), washed with water and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL® Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the HISP1 gene as a probe. Test compounds resulting in a reduced level of HISP1 mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Example 40

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Histidinol-phosphatase with No Activity

Magnaporthe grisea fungal cells containing a mutant form of the HISP1 gene which abolishes enzyme activity, such as a gene containing a transposon insertion (see

Examples 32 and 33), are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-histidine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-histidine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 41

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Histidinol-phosphatase with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of the HISP1 gene, such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-histidine (Sigma-Aldrich Co.) after growth

for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 42

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-histidine Biosynthetic Gene with No Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-histidine biosynthetic pathway (*e.g.* Histidinol dehydrogenase (E.C. 1.1.1.23)) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-histidine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 µM L-histidine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound

present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 43

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-histidine Biosynthetic Gene with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-histidine biosynthetic pathway (*e.g.* Histidinol dehydrogenase (E.C. 1.1.1.23)), such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989).Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-histidine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without

cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 44

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Fungal HISP1 and a Second Fungal Strain Containing a Heterologous HISP1 Gene

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional HISP1 gene and containing a heterologous HISP1 gene are grown under standard fungal growth conditions that are well known and described in the art. A *M. grisea* strain carrying a heterologous HISP1 gene is made as follows:

- A *M. grisea* strain is made with a nonfunctional HISP1 gene, such as one containing a transposon insertion in the native gene (see Examples 32 and 33).
- A construct containing a heterologous HISP1 gene is made by cloning the heterologous HISP1 gene into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll *et al.* (1994) Fungal Gen News Lett *41*: 22) using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press).
- The said construct is used to transform the *M. grisea* strain lacking a functional HISP1 gene (see Example 33). Transformants are selected on minimal agar medium lacking L-histidine. Only transformants carrying a functional HISP1 gene will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of HISP1 are grown under standard fungal growth conditions that are well known and

described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous HISP1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 45

Pathway Specific In Vivo Assay Screening Protocol

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing 4 mM L-histidine (Sigma-Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 35). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well

containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing 4 mM L-histidine. Test compounds are added to wells containing spores in minimal media and minimal media containing L-histidine. The total volume in each well is 200µl. Both minimal media and L-histidine containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the L-histidine biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well containing L-histidine as a result of the addition of the test compound. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 46

High Throughput Preparation and Verification of Transposon Insertion into the M. grisea IPMD1 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) supplemented with 50 ug/ml of ampicillin. Blocks were incubated with shaking at 37 C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.* (1997) Genome Res 7: 1072 – 84 (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* IPMD1 gene was chosen for further analysis. This construct was designated cpgmra0014081f03 and it contains the SIF transposon approximately between

amino acids 560 and 561 relative to the *Rhizomucor pusillus* homologue LeuA (total length: 755 amino acids, SWISS-PROT: P55251).

Example 47

Preparation of IPMD1 Cosmid DNA and Transformation of Magnaporthe grisea

Cosmid DNA from the IPMD1 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (QIAGEN), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.* (1997) MPMI *10*: 700 - 708). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) shaking at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of $2x10^8$ protoplasts/ml. 50ul protoplast suspension was mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose for one day, then overlayed with CM agar media containing hygromycin B (250ug/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)). Two independent strains were identified and are hereby referred to as KO1-3 and KO1-7, respectively.

Example 48

Effect of Transposon Insertion on Magnaporthe pathogenicity

The target fungal strains, KO1-3 and KO1-7, obtained in Example 47and the wild type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-week period. Rice infection assays were performed using Indian rice cultivar CO39 essentially as described in Valent *et al.* ((1991) Genetics *127*: 87 - 101 (PMID: 2016048)). All three strains were grown for spore production on complete agar media.

Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of conidial suspension (5 x 10^4 conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of IPMD1 gene disruption on *Magnaporthe* infection at five days post-inoculation.

Example 49

Verification of IPMD1 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-3 and KO1-7, containing the IPMD1 disrupted gene obtained in Example 47 were analyzed for their nutritional requirement for L-leucine using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The innoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$, 6.7mM KCl, 3.5mM Na_2SO_4, 11mM KH_2PO_4, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM $MgCl_2$, 1.0mM $CaCl_2$ and trace elements. Final concentrations of trace elements are: 7.6μM $ZnCl_2$, 2.5μM $MnCl_2{\cdot}4H_2O$, 1.8μM $FeCl_2{\cdot}4H_2O$, 0.71μM $CoCl_2{\cdot}6H_2O$, 0.64μM $CuCl_2{\cdot}2H_2O$, 0.62μM Na_2MoO_4, 18μM H_3BO_3. pH adjusted to 6.0 with NaOH. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to 2x10^5 spores/ml. 100μl of spore suspension were deposited into each well of the microtiter plates. The plates were incubated at 25°C for 7 days. Optical density (OD) measurements at 490nm and 750nm were taken daily. The OD_{490} measures the extent of tetrazolium dye reduction and the level of growth, and OD_{750} measures growth only. Turbidity = OD_{490} + OD_{750}. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of L-leucine.

Example 50

Cloning and Expression Strategies, Extraction and Purification of 3-Isopropylmalate Dehydratase Protein.

The following protocol may be employed to obtain a purified 3-Isopropylmalate dehydratase protein.

Cloning and expression strategies:

An IPMD1 cDNA gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein can be evaluated by SDS-PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer by sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 51

Assays for Testing Binding of Test Compounds to 3-Isopropylmalate Dehydratase

The following protocol may be employed to identify test compounds that bind to the 3-Isopropylmalate dehydratase protein.

- Purified full-length 3-Isopropylmalate dehydratase polypeptide with a His/fusion protein tag (Example 50) is bound to a HisGrab™ Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, as may be described in Satyanarayana *et al.* ((1968B) J Bacteriol *96*: 2018 – 24 (PMID: 5724970)) and/or Kohlhaw ((1988) Methods Enzymol *166*: 423 - 9 (PMID: 3071717))) for binding of radiolabeled 2-Isopropylmalate (custom made PerkinElmer Life Sciences, Inc., Boston, MA) to the bound 3-Isopropylmalate dehydratase.
- Screening of test compounds is performed by adding test compound and 2-Isopropylmalate (custom made PerkinElmer Life Sciences, Inc., Boston, MA) to the wells of the HisGrab™ plate containing bound 3-Isopropylmalate dehydratase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (Scintiverse®, Fisher Scientific) is added to each well.
- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* 3-Isopropylmalate dehydratase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the IPMD1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 50). Oligonucleotide primers are designed to amplify a portion of the IPMD1 gene using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 50 above.

Test compounds that bind IPMD1 are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2×10^5 spores/ml and the culture is divided. The test compound is

added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 52

Assays for Testing Inhibitors or Candidates for Inhibition of 3-Isopropylmalate Dehydratase Activity

The enzymatic activity of 3-Isopropylmalate dehydratase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Satyanarayana *et al.* ((1968B) J Bacteriol *96*: 2018 – 24 (PMID: 5724970)) and/or Kohlhaw ((1988) Methods Enzymol *166*: 423 - 9 (PMID: 3071717)). Candidate compounds are identified when a decrease in products or a lack of decrease in substrates is detected with the reaction proceeding in either direction.

Additionally, the enzymatic activity of a polypeptide comprising 10-50 amino acids from the *M. grisea* 3-Isopropylmalate dehydratase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Satyanarayana *et al.* ((1968B) J Bacteriol *96*: 2018 – 24 (PMID: 5724970)) and/or Kohlhaw ((1988) Methods Enzymol *166*: 423 - 9 (PMID: 3071717)). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the IPMD1 gene into a protein expression vector that adds a His-Tag when expressed (see Example 50). Oligonucleotide primers are designed to amplify a portion of the IPMD1 gene using polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed and purified as described in Example 50 above.

Test compounds identified as inhibitors of IPMD1 activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*:

1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 53

Assays for Testing Compounds for Alteration of 3-Isopropylmalate Dehydratase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem®, La Jolla, CA), washed with water and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL® Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the IPMD1 gene as a probe. Test compounds resulting in a reduced level of IPMD1 mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Example 54

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of 3-Isopropylmalate dehydratase with No Activity

Magnaporthe grisea fungal cells containing a mutant form of the IPMD1 gene which abolishes enzyme activity, such as a gene containing a transposon insertion (see Examples 46 and 47), are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-leucine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-leucine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 55

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of 3-Isopropylmalate dehydratase with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of the IPMD1 gene, such as a promoter truncation that reduces expression, are grown under standard fungal

growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-leucine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 56

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-leucine Biosynthetic Gene with No Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-leucine biosynthetic pathway (*e.g.* a 3-Isopropylmalate dehydrogenase (E.C. 1.1.1.85)) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-leucine (Sigma-Aldrich Co.) after growth for 10-13 days in

the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-leucine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 57

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-leucine Biosynthetic Gene with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-leucine biosynthetic pathway (*e.g.* a 3-Isopropylmalate dehydrogenase (E.C. 1.1.1.85)), such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-leucine (Sigma-Aldrich Co.) after growth for 10-13 days in

the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 58

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Fungal IPMD1 and a Second Fungal Strain Containing a Heterologous IPMD1 Gene

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional IPMD1 gene and containing a 3-isopropylmalate dehydratase large subunit gene from *Xylella fastidiosa* (Genbank accession number H82564, 63% sequence identity) are grown under standard fungal growth conditions that are well known and described in the art. A *M. grisea* strain carrying a heterologous IPMD1 gene is made as follows:

- A *M. grisea* strain is made with a nonfunctional IPMD1 gene, such as one containing a transposon insertion in the native gene (see Examples 46 and 47).
- A construct containing a heterologous IPMD1 gene is made by cloning the 3-isopropylmalate dehydratase large subunit gene from *Xylella fastidiosa* into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll

et al. (1994) Fungal Gen News Lett *41*: 22) using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press).

- The said construct is used to transform the *M. grisea* strain lacking a functional IPMD1 gene (see Example 47). Transformants are selected on minimal agar medium lacking L-leucine. Only transformants carrying a functional IPMD1 gene will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of IPMD1 are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous IPMD1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 59

Pathway Specific In Vivo Assay Screening Protocol

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are

harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing 4 mM L-leucine (Sigma-Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 49). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing 4 mM L-leucine. Test compounds are added to wells containing spores in minimal media and minimal media containing L-leucine. The total volume in each well is 200µl. Both minimal media and L-leucine containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the L-leucine biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well containing L-leucine as a result of the addition of the test compound. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 60

High Throughput Preparation and Verification of Transposon Insertion into the M. grisea THR4 Gene

E. coli strains containing cosmids with transposon insertions were picked to 96 well growth blocks (Beckman Co.) containing 1.5 ml of TB (Terrific Broth, Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) supplemented with 50 ug/ml of ampicillin. Blocks were incubated with shaking at 37°C overnight. *E. coli* cells were pelleted by centrifugation and cosmids were isolated by a modified alkaline lysis method (Marra *et al.* (1997) Genome Res *7*: 1072 – 84 (PMID: 9371743)). DNA quality was checked by electrophoresis on agarose gels. Cosmids were sequenced using primers from the ends of each transposon and commercial

dideoxy sequencing kits (Big Dye Terminators, Perkin Elmer Co.). Sequencing reactions were analyzed on an ABI377 DNA sequencer (Perkin Elmer Co.).

DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* THR4 gene was chosen for further analysis. This construct was designated cpgmra0012020a04 and it contains the SIF transposon approximately between amino acids 314 and 315 relative to the *Schizosaccharomyces pombe* homologue ThrC (total length: 514 amino acids, GENBANK: 2501152).

Example 61

Preparation of THR4 Cosmid DNA and Transformation of Magnaporthe grisea

Cosmid DNA from the THR4 transposon tagged cosmid clone was prepared using QIAGEN Plasmid Maxi Kit (QIAGEN), and digested by PI-PspI (New England Biolabs, Inc.). Fungal electro-transformation was performed essentially as described (Wu *et al.* (1997) MPMI *10*: 700 - 708). Briefly, *M. grisea* strain Guy 11 was grown in complete liquid media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) shaking at 120 rpm for 3 days at 25°C in the dark. Mycelia was harvested and washed with sterile H_2O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of $2x10^8$ protoplasts/ml. 50ul protoplast suspension was mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose for one day, then overlayed with CM agar media containing hygromycin B (250ug/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA *98*: 5110 – 15 (PMID: 11296265)). Two independent strains were identified and are hereby referred to as KO1-3 and KO1-22, respectively.

Example 62

Effect of Transposon Insertion on Magnaporthe pathogenicity

The target fungal strains, KO1-3 and KO1-22, obtained in Example 61 and the wild type strain, Guy11, were subjected to a pathogenicity assay to observe infection over a 1-week period. Rice infection assays were performed using Indian rice cultivar CO39 essentially as described in Valent *et al.* ((1991) Genetics *127*: 87 - 101 (PMID: 2016048)). All three strains were grown for spore production on complete agar media. Spores were harvested and the concentration of spores adjusted for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed with 12 ml of conidial suspension (5 x 10^4 conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27 °C 12 hours/21 °C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of THR4 gene disruption on *Magnaporthe* infection at five days post-inoculation.

Example 63

Verification of THR4 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-3 and KO1-22, containing the THR4 disrupted gene obtained in Example 61 were analyzed for their nutritional requirement for L-threonine using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The innoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$, 6.7mM KCl, 3.5mM Na_2SO_4, 11mM KH_2PO_4, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM $MgCl_2$, 1.0mM $CaCl_2$ and trace elements, pH adjusted to 6.0 with NaOH. Final concentrations of trace elements are: 7.6μM $ZnCl_2$, 2.5μM $MnCl_2 \cdot 4H_2O$, 1.8μM $FeCl_2 \cdot 4H_2O$, 0.71μM $CoCl_2 \cdot 6H_2O$, 0.64μM $CuCl_2 \cdot 2H_2O$, 0.62μM Na_2MoO_4, 18μM H_3BO_3. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to 2x10^5 spores/ml. 100μl of spore suspension were deposited into each well of the microtiter plates. The plates were incubated at 25°C for 7

days. Optical density (OD) measurements at 490nm and 750nm were taken daily. The OD_{490} measures the extent of tetrazolium dye reduction and the level of growth, and OD_{750} measures growth only. Turbidity = OD_{490} + OD_{750}. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 3A) and presence (Figure 3B) of L-threonine.

Example 64

Cloning and Expression Strategies, Extraction and Purification of Threonine Synthase Protein.

The following protocol may be employed to obtain a purified Threonine synthase protein.

Cloning and expression strategies:

A THR4 cDNA gene can be cloned into *E. coli* (pET vectors-Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags, and the expression of recombinant protein can be evaluated by SDS-PAGE and Western blot analysis.

Extraction:

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer by sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

Purification:

Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer
- Elute bound protein with 0.5 M imidazole

Example 65

Assays for Testing Binding of Test Compounds to Threonine Synthase

The following protocol may be employed to identify test compounds that bind to the Threonine synthase protein.

- Purified full-length Threonine synthase polypeptide with a His/fusion protein tag (Example 64) is bound to a HisGrab™ Nickel Coated Plate (Pierce, Rockford, IL) following manufacturer's instructions.
- Buffer conditions are optimized (*e.g.* ionic strength or pH, Ramos and Calderon (1994) FEBS Lett *351*: 357 - 9 (PMID: 8082795)) for binding of radiolabeled O-phospho-L-homoserine (Gening *et al.* (1994) Biokhimiia *59*: 1238 - 44 (PMID: 7819407)) to the bound Threonine synthase.
- Screening of test compounds is performed by adding test compound and radiolabeled O-phospho-L-homoserine (Gening *et al.* (1994) Biokhimiia *59*: 1238 - 44 (PMID: 7819407)) to the wells of the HisGrab™ plate containing bound Threonine synthase.
- The wells are washed to remove excess labeled ligand and scintillation fluid (Scintiverse®, Fisher Scientific) is added to each well.
- The plates are read in a microplate scintillation counter.
- Candidate compounds are identified as wells with lower radioactivity as compared to control wells with no test compound added.

Additionally, a purified polypeptide comprising 10-50 amino acids from the *M. grisea* Threonine synthase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the THR4 gene into a protein expression vector that adds a His-Tag when expressed (see Example 64). Oligonucleotide primers are designed to amplify a portion of the THR4 gene using the polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed in a host organism and purified as described in Example 64 above.

Test compounds that bind THR4 are further tested for antibiotic activity. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal

media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 66

Assays for Testing Inhibitors or Candidates for Inhibition of Threonine Synthase Activity

The enzymatic activity of Threonine synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Ramos and Calderon (1994) FEBS Lett *351*: 357 - 9 (PMID: 8082795). Candidate compounds are identified when a decrease in products or a lack of decrease in substrates is detected with the reaction proceeding in either direction.

Additionally, the enzymatic activity of a polypeptide comprising 10-50 amino acids from the *M. grisea* Threonine synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Ramos and Calderon (1994) FEBS Lett *351*: 357 - 9 (PMID: 8082795). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the THR4 gene into a protein expression vector that adds a His-Tag when expressed (see Example 64). Oligonucleotide primers are designed to amplify a portion of the THR4 gene using polymerase chain reaction amplification method. The DNA fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression vector, expressed and purified as described in Example 64 above.

Test compounds identified as inhibitors of THR4 activity are further tested for antibiotic activity. *Magnaporthe grisea* fungal cells are grown under standard fungal growth conditions that are well known and described in the art. *M. grisea* is grown as described for spore production on oatmeal agar media (Talbot *et al.* (1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)). Spores are harvested into minimal media (Talbot *et al.*

(1993) Plant Cell *5*: 1575 - 1590 (PMID: 8312740)) to a concentration of 2 x 10^5 spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100 μg/ml. Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

Example 67

Assays for Testing Compounds for Alteration of Threonine Synthase Gene Expression

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on complete agar or oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of 2x10^5 spores per ml. 25 ml cultures are prepared to which test compounds will be added at various concentrations. A culture with no test compound present is included as a control. The cultures are incubated at 25°C for 3 days after which test compound or solvent only control is added. The cultures are incubated an additional 18 hours. Fungal mycelia is harvested by filtration through Miracloth (CalBiochem®, La Jolla, CA), washed with water and frozen in liquid nitrogen. Total RNA is extracted with TRIZOL® Reagent using the methods provided by the manufacturer (Life Technologies, Rockville, MD). Expression is analyzed by Northern analysis of the RNA samples as described (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the THR4 gene as a probe. Test compounds resulting in a reduced level of THR4 mRNA relative to the untreated control sample are identified as candidate antibiotic compounds.

Example 68

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Threonine Synthase with No Activity

Magnaporthe grisea fungal cells containing a mutant form of the THR4 gene which abolishes enzyme activity, such as a gene containing a transposon insertion (see Examples 60 and 61), are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-threonine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-threonine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

Example 69

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of Threonine Synthase with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of the THR4 gene, such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is

made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-threonine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores are added to each well of 96-well plates to which a test compound is added (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 70

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-threonine Biosynthetic Gene with No Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-threonine biosynthetic pathway (*e.g.* Homoserine kinase (E.C. 2.7.1.39)) are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-threonine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined

using a hemacytometer and spore suspensions are prepared in a minimal growth medium containing 100 μM L-threonine to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200μl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild-type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 71

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of a L-threonine Biosynthetic Gene with Reduced Activity

Magnaporthe grisea fungal cells containing a mutant form of a gene in the L-threonine biosynthetic pathway (*e.g.* Homoserine kinase (E.C. 2.7.1.39)), such as a promoter truncation that reduces expression, are grown under standard fungal growth conditions that are well known and described in the art. A promoter truncation is made by deleting a portion of the promoter upstream of the transcription start site using standard molecular biology techniques that are well known and described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 4 mM L-threonine (Sigma-Aldrich Co.) after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is

determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 72

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Fungal THR4 and a Second Fungal Strain Containing a Heterologous THR4 Gene

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional THR4 gene and containing a Thr4 gene from *Saccharomyces cerevisiae* (Genbank: 6319901, 50% sequence identity) are grown under standard fungal growth conditions that are well known and described in the art. A *M. grisea* strain carrying a heterologous THR4 gene is made as follows:

- A *M. grisea* strain is made with a nonfunctional THR4 gene, such as one containing a transposon insertion in the native gene (see Examples 60 and 61).
- A construct containing a heterologous THR4 gene is made by cloning the Thr4 gene from *Saccharomyces cerevisiae* into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll *et al.* (1994) Fungal Gen News Lett *41*: 22) using standard molecular biology techniques that are well known and

described in the art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual).

- The said construct is used to transform the *M. grisea* strain lacking a functional THR4 gene (see Example 61). Transformants are selected on minimal agar medium lacking L-threonine. Only transformants carrying a functional THR4 gene will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of THR4 are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined using a hemacytometer and spore suspensions are prepared in a minimal growth medium to a concentration of $2x10^5$ spores per ml. Approximately $4x10^4$ spores or cells are harvested and added to each well of 96-well plates to which growth media is added in addition to an amount of test compound (at varying concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the OD_{590} (fungal strain plus test compound) / OD_{590} (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous THR4 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

Example 73

Pathway Specific In Vivo Assay Screening Protocol

Magnaporthe grisea fungal cells are grown under standard fungal growth conditions that are well known and described in the art. Wild-type *M. grisea* spores are harvested from cultures grown on oatmeal agar media after growth for 10-13 days in the light at 25°C using a moistened cotton swab. The concentration of spores is determined

using a hemocytometer and spore suspensions are prepared in a minimal growth medium and a minimal growth medium containing 4 mM L-threonine (Sigma-Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium, and trace elements (for example, see innoculating fluid in Example 63). Spore suspensions are added to each well of a 96-well microtiter plate (approximately $4x10^4$ spores/well). For each well containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium containing 4 mM L-threonine. Test compounds are added to wells containing spores in minimal media and minimal media containing L-threonine. The total volume in each well is 200µl. Both minimal media and L-threonine containing media wells with no test compound are provided as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. A compound is identified as a candidate for an antibiotic acting against the L-threonine biosynthetic pathway when the observed growth in the well containing minimal media is less than the observed growth in the well containing L-threonine as a result of the addition of the test compound. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221).

While the foregoing describes certain embodiments of the invention, it will be understood by those skilled in the art that variations and modifications may be made and still fall within the scope of the invention. The foregoing examples are intended to exemplify various specific embodiments of the invention and do not limit its scope in any manner.

SEQUENCE LISTING

<110> Lo, Sze Chung C

Montenegro-Chamorro, Maria V

Frank, Sheryl A

Darveaux, Blaise A

Mahanty, Sanjoy K

Heiniger, Ryan W

Skalchunes, Amy R

Pan, Huaqin

Tarpey, Rex

Shuster, Jeffrey R

Tanzer, Matthew M

Hamer, Lisbeth

Adachi, Kiichi

DeZwaan, Todd M

<120> METHODS FOR THE IDENTIFICATION OF INHIBITORS OF ASPARAGINE SYNTHASE, 5-AMINOLEVULINATE SYNTHASE, HISTIDINOL-PHOSPHATASE, 3-ISOPROPYLMALATE AND THREONINE SYNTHASE AS ANTIBIOTICS

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tgttctctgg gccattgata ctcactgcca tcaccaattg gatctacccc aactactata 180

ctttgacacc cccccaaaaa aaaacccttt aaaacgaagt cacaatgtgt ggcatcttcg 240

cctgccacgc acacccggat gtgcaaaaat tcaagcccac cgccctccgg ctcgcaaaag 300

cgatccgaca tcggggtccc gattggagcg gaagcgtgac ttgcaaccag acgatattgt 360

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gcttgaagac cccataccac ttcaagacaa catccgactg cgaggtcatc atccctctgg 540

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ccaacatggt gggacccaaa gagggtaccg gaaacaccac tcgatctcac aaagttgcgc 960

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gagaaggaaa ggaaattttc agggggctta gagtagatgg atgttaatag aaaccagaca 2220

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atgtgagatc cgggcttttt t 2361

<210> 3

<211> 589

<212> PRT

<213> Magnaporthe grisea

<400> 3

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Lys Pro Thr Ala Leu Arg Leu Ala Lys Ala Ile Arg His Arg Gly Pro
20 25 30

Asp Trp Ser Gly Ser Val Thr Cys Asn Gln Thr Ile Leu Cys His Glu
35 40 45

Arg Leu Ser Ile Val Gly Val Glu Ser Gly Ala Gln Pro Leu Thr Asn
50 55 60

Glu Asp Glu Ser Ile Ile Leu Ala Val Asn Gly Glu Ile Tyr Asn His
65 70 75 80

Arg Leu Val Arg Lys Ser Leu Lys Thr Pro Tyr His Phe Lys Thr Thr
85 90 95

Ser Asp Cys Glu Val Ile Ile Pro Leu Tyr Met Glu His Gly Leu Asp
100 105 110

Ala Pro Lys Tyr Leu Asp Gly Met Phe Ser Phe Val Leu Tyr Asp Lys
115 120 125

Lys Gln Asn Arg Thr Ile Ala Ala Arg Asp Pro Ile Gly Val Thr Thr
130 135 140

Phe Tyr Gln Gly Trp Ser Ser Ser Glu Pro Gly Ala Val Tyr Phe Gly
145 150 155 160

Ser Glu Leu Lys Cys Leu Tyr Gln Val Cys Asp Lys Ile Glu Ala Phe
165 170 175

Pro Pro Gly His Ile Tyr Asp Ser Leu Thr Gly Glu Arg Thr Arg Tyr
180 185 190

Phe Gln Pro Thr Trp Trp Asp Pro Lys Arg Val Pro Glu Thr Pro Leu
195 200 205

Asp Leu Thr Lys Leu Arg Glu Ala Leu Glu Lys Ser Val Arg Lys Arg
210 215 220

Leu Met Ala Glu Val Pro Tyr Gly Val Leu Leu Ser Gly Gly Leu Asp
225 230 235 240

Ser Ser Leu Val Ala Ser Ile Ala Gln Arg Glu Thr Lys Arg Leu Lys
245 250 255

Lys Leu Ala Ile Glu Ala Gly Leu Glu Asp Leu Pro Ala Glu Pro Thr
260 265 270

Gly Asn His Asp Gln Gly Glu Gly Leu Val Gly Ile Asp Asp Glu Asn
275 280 285

Lys Leu Ser Thr Met Thr Tyr Leu Pro Gln Leu Asn Ser Phe Ser Ile
290 295 300

Gly Leu Pro Gly Ser Pro Asp Asn Lys Ala Ala Leu Glu Val Ala Lys
305 310 315 320

Phe Leu Gly Thr Lys His His Val Met Thr Phe Thr Ile Glu Asp Gly
325 330 335

Leu Asn Ala Leu Ser Asp Val Ile Tyr His Leu Glu Ser Tyr Asp Val
340 345 350

Thr Thr Ile Arg Ala Ser Thr Pro Met Tyr Leu Leu Ser Arg Lys Ile
355 360 365

Lys Ala Met Gly Ile Lys Met Val Leu Ser Gly Glu Gly Ser Asp Glu
370 375 380

Ala Phe Gly Gly Tyr Leu Tyr Phe His Asn Ala Pro Asp Lys Asp Ala
385 390 395 400

Phe His Asp Glu Thr Val Arg Arg Val Lys Asn Leu His Leu Ser Asp
405 410 415

Cys Leu Arg Ala Asn Lys Ser Thr Ser Ala Trp Gly Leu Glu Ala Arg
420 425 430

Val Pro Phe Leu Asp Lys Glu Phe Leu Glu Leu Ala Met Asn Ile Asp
435 440 445

Pro Lys Glu Lys Met Ile Thr Lys Glu Arg Ile Glu Lys Tyr Ile Val
450 455 460

Arg Lys Ala Phe Asp Thr Ser Asp Asp Pro Asn Ala Glu Pro Tyr Leu
465 470 475 480

Pro Asp Asn Ile Leu Trp Arg Gln Lys Glu Gln Phe Ser Asp Gly Val
485 490 495

Gly Tyr Gly Trp Ile Asp Ala Leu Lys Asp Asn Ala Glu Ile Gln Val
500 505 510

Thr Asp Glu Met Met Lys Asn Pro Lys Pro Glu Trp Gly Asp Asp Ile
515 520 525

Pro Asp Thr Lys Glu Ala Tyr Trp Tyr Arg Cys Met Phe Asp Glu His
530 535 540

Phe Pro Ser Thr Leu Arg Leu Asp Trp Trp Ser Ala Gly Pro Arg Arg
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Gly Leu Ser Arg Pro Ile Pro Val Ala Glu Pro Ser Arg Ser Met Leu
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Pro Ser Met Thr Thr Ser Ala Ser Asn Gly Thr Thr Asn
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<210> 4

<211> 1848

<212> DNA

<213> Magnaporthe grisea

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ggtggtacca tctccaagct gcagctgctg gcgcaccggt gccccgtcat gggccaggca 180

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gtgcccttcc cgcccaaggc tgcttccggc cgatccgccg cctcgtcgaa ccccgccgcg 360

aacgcctccc cggccgccgg ccacccgggc aagaagtttt cctatgagcg attctacgaa 420

tccgaactgc agaagaagca caaggacaaa tcgtaccgct acttcaacaa catcaaccgc 480

ctggccaagg agttcccccg cgctcacatg tcggacaagg aggacaaggt gaccgtttgg 540

tgcgccaacg actacctggg catgggccgc aaccccaggg tcttgtccaa gatgcacgag 600

acgctcgatg agtacggtgc tggcgccggt ggtactcgca acatctctgg ccacaaccgc 660

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gattgtgtga tcctgtcgga cagcctaaac catgcttcca tgatccaggg tatccgccat 840

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gatattatca ccggaacgct tggcaaagct tacggctgcg ttggcggcta catcgccggt 1200

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<210> 5

<211> 2275

<212> DNA

<213> Magnaporthe grisea

<400> 5

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aggccttgtg ccccttcctc aagaaggcgt cgccggccac tctgcggcag ctctcgacag 180

catctgcacc ggccaggccc cgtgtgtcac catgtggtgg taccatctcc aagctgcagc 240

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gtacgaagct gcccgtcggc ctggccaaga tacacacttc gcggagccag gatgctaggg 360

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agaatagtgc ccttcccgcc caaggctgct tccggccgat ccgccgcctc gtcgaacccc 540

gccgcgaacg cctccccggc cgccggccac ccgggcaaga agttttccta tgagcgattc 600

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<210> 6

<211> 615

<212> PRT

<213> Magnaporthe grisea

<400> 6

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Lys Ala Ser Pro Ala Thr Leu Arg Gln Leu Ser Thr Ala Ser Ala Pro
20 25 30

Ala Arg Pro Arg Val Ser Pro Cys Gly Gly Thr Ile Ser Lys Leu Gln
35 40 45

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Leu Leu Ala His Arg Cys Pro Val Met Gly Gln Ala Met Ala Val Gln
    50                  55                  60

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

Thr Ser Arg Ser Gln Asp Ala Arg Ala Val Asp Gly Pro Val Val Ala
                85                  90                  95

Ser Arg Glu Asn Val Pro Phe Pro Pro Lys Ala Ala Ser Gly Arg Ser
            100                 105                 110

Ala Ala Ser Ser Asn Pro Ala Ala Asn Ala Ser Pro Ala Ala Gly His
        115                 120                 125

Pro Gly Lys Lys Phe Ser Tyr Glu Arg Phe Tyr Glu Ser Glu Leu Gln
    130                 135                 140

Lys Lys His Lys Asp Lys Ser Tyr Arg Tyr Phe Asn Asn Ile Asn Arg
145                 150                 155                 160

Leu Ala Lys Glu Phe Pro Arg Ala His Met Ser Asp Lys Glu Asp Lys
                165                 170                 175

Val Thr Val Trp Cys Ala Asn Asp Tyr Leu Gly Met Gly Arg Asn Pro
            180                 185                 190

Arg Val Leu Ser Lys Met His Glu Thr Leu Asp Glu Tyr Gly Ala Gly
        195                 200                 205

Ala Gly Gly Thr Arg Asn Ile Ser Gly His Asn Arg His Ala Val Glu
    210                 215                 220

Leu Glu Gly Thr Ile Ala Lys Leu His Ala Lys Asp Ser Ala Leu Val
225                 230                 235                 240

Phe Ser Ser Cys Tyr Val Ala Asn Asp Ala Thr Leu Ala Thr Leu Gly
                245                 250                 255

Ser Lys Met Pro Asp Cys Val Ile Leu Ser Asp Ser Leu Asn His Ala
            260                 265                 270
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Ser Met Ile Gln Gly Ile Arg His Ser Gly Thr Lys Lys Ile Val Phe
275 280 285

Lys His Asn Asp Val Ala Asp Leu Glu Ala Lys Leu Ala Ser Leu Pro
290 295 300

Leu His Val Pro Lys Ile Ile Ala Phe Glu Ser Val Tyr Ser Met Cys
305 310 315 320

Gly Ser Ile Gly Pro Ile Glu Glu Ile Cys Asp Leu Ala Asp Lys Tyr
325 330 335

Gly Ala Ile Thr Phe Leu Asp Glu Val His Ala Val Gly Met Tyr Gly
340 345 350

Pro His Gly Ala Gly Val Ala Glu His Leu Asp Phe Glu Ala His Lys
355 360 365

Ala Gly Arg Pro Arg Gly Thr Ile Met Asp Arg Ile Asp Ile Ile Thr
370 375 380

Gly Thr Leu Gly Lys Ala Tyr Gly Cys Val Gly Gly Tyr Ile Ala Gly
385 390 395 400

Ser Ala Lys Leu Ile Asp Met Ile Arg Ser Leu Ala Pro Gly Phe Ile
405 410 415

Phe Thr Thr Ser Leu Pro Pro Ala Thr Met Ala Gly Ala Arg Ala Ala
420 425 430

Ile Glu Tyr Gln Met Glu His Asp Gly Asp Arg Arg Leu Gln Gln Leu
435 440 445

His Thr Arg Ala Val Lys Glu Ala Leu Gln His Arg Asp Ile Pro Val
450 455 460

Ile Pro Asn Pro Ser His Ile Ile Pro Ile Leu Val Gly Asn Ala Glu
465 470 475 480

Leu Ala Lys Arg Ala Ser Asp Met Leu Leu Ser Asp Tyr Gln Ile Tyr
485 490 495

Val Gln Ser Ile Asn Tyr Pro Thr Val Pro Val Gly Gln Glu Arg Leu
500 505 510

Arg Val Thr Pro Thr Pro Gly His Val Lys Glu Phe Arg Asp Asp Leu
515 520 525

Val Val Ala Val Asp Ala Ile Trp Thr Lys Leu Gly Ile Lys Arg Thr
530 535 540

Ser Glu Trp Ala Ala Glu Gly Gly Phe Ile Gly Val Gly Glu Glu Gly
545 550 555 560

Ser Glu Ala Gln Ala Gln Pro Leu Trp Thr Asp Ala Gln Leu Gly Ile
565 570 575

Glu Gln Ala Ala Lys Glu Ile Met Ala Leu Gly Thr Ala Pro Thr Gly
580 585 590

Cys Phe Thr Glu Ser Leu Ile Glu Arg Glu Gly Ala Ala Leu Gly Arg
595 600 605

Gly Ser Met Ala Ala Ala Ala
610 615

<210> 7

<211> 1002

<212> DNA

<213> Magnaporthe grisea

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cacatgccac ggacccaact gtgcgatcta tatccagaag aacttgtgcc tgacccgcac 180

gcctccctcg cggagctgat gccgcgccac gctgcctaca tgaccgaggc gcgtcggctg 240

caaaagaagt acgccgatcg catcaccctc ctcatcggct tcgagggcga gttcatccgg 300

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gcgctccgca aggggctcgc cgaaccgtac ccgtgccggc ccatcgcgga ggaatgggta 780

aggctgggcg gtaagttcac aatgtctgac gacagccacg gcatcgcgca ggttgccaca 840

aactatgtgc gagccctgga ctacctcgag tcgctcggcg tgaacgaggt ctggacgtat 900

gaccgagcta aagagggatc agagcttgtg gagaagggtg tgtcgtttac agagtttcgc 960

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<210> 8

<211> 1717

<212> DNA

<213> Magnaporthe grisea

<400> 8

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cattaatgga gcaagtaaaa cgtattcaag cctccgagga cccagcagcc gccctcgcgc 180

cccgaaatac gcgcgctttc aggcattgct tgagcctatc tttggtgggg ttacacttta 240

aaaaaagttg ttcctgcgtg cctcccatcc aatgcctggc atccatctta gctgaggcag 300

gtaccagaca cctgggtaat gaatgctaag agtcaatctc tctaggaatt taacggattt 360

gattggctag caaacattga ttgatttttc ttcgtacatt tccatgtacg ccgtatagca 420

ttacaaagag taaaagcaag tttggcctca gttaccctaa atccagatac agacagacag 480

acgccctccc tatctgcctt gaatggttga agttttaata gccggcatcc ctccgtttcc 540

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catttttttt ccatcgcaaa agcaaacaca cagtcatggc cttcacaatg cactcgcact 660

ccgggcaatt ctgcccgggc cacgcaaaag accagttgga agacgtcatc ctccatgcca 720

tcagcatagg atacaagacc atgggtctca gtgagcacat gccacggacc caactgtgcg 780

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<210> 9

<211> 333

<212> PRT

<213> Magnaporthe grisea

<400> 9

Met Ala Phe Thr Met His Ser His Ser Gly Gln Phe Cys Pro Gly His
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Ala Lys Asp Gln Leu Glu Asp Val Ile Leu His Ala Ile Ser Ile Gly
20 25 30

Tyr Lys Thr Met Gly Leu Ser Glu His Met Pro Arg Thr Gln Leu Cys
35 40 45

Asp Leu Tyr Pro Glu Glu Leu Val Pro Asp Pro His Ala Ser Leu Ala
50 55 60

Glu Leu Met Pro Arg His Ala Ala Tyr Met Thr Glu Ala Arg Arg Leu
65 70 75 80

Gln Lys Lys Tyr Ala Asp Arg Ile Thr Leu Leu Ile Gly Phe Glu Gly
85 90 95

Glu Phe Ile Arg Ser Glu Tyr Gly Thr Leu Val Arg Ser Leu Ala Asp
100 105 110

Gly Asn Gly Asp Pro Ser Tyr Phe Gln Asn Gly Asp Ser Lys Leu Val
115 120 125

Thr Asp Ala Gly Lys Val Asp Tyr Phe Ile Gly Ser Leu His His Gly
130 135 140

Ala Gly Gly Ile Pro Ile Asp Phe Asp Arg Ala Thr Tyr Leu Arg Ser
145 150 155 160

Val Glu Ala Ala Gly Pro Asn Gly Glu Glu Asp Leu Phe Val His Tyr
165 170 175

Tyr Asp Gln Gln Phe Glu Met Leu Gln Ala Leu Arg Pro Pro Ile Val
180 185 190

Gly His Phe Asp Leu Ile Arg Leu Met Ser Glu Glu Pro Gly Arg Asn
195 200 205

Pro Ser Ala Trp Ser Pro Asn Arg Val Trp Pro Leu Ile Lys Arg Asn
210 215 220

Leu Ala Phe Val Ala Ser Tyr Gly Gly Trp Leu Glu Cys Asn Ser Ser
225 230 235 240

Ala Leu Arg Lys Gly Leu Ala Glu Pro Tyr Pro Cys Arg Pro Ile Ala
245 250 255

Glu Glu Trp Val Arg Leu Gly Gly Lys Phe Thr Met Ser Asp Asp Ser
260 265 270

His Gly Ile Ala Gln Val Ala Thr Asn Tyr Val Arg Ala Leu Asp Tyr
275 280 285

Leu Glu Ser Leu Gly Val Asn Glu Val Trp Thr Tyr Asp Arg Ala Lys
290 295 300

Glu Gly Ser Glu Leu Val Glu Lys Gly Val Ser Phe Thr Glu Phe Arg
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Gly Ser Leu Arg Leu Pro Thr Thr Ala Ser Lys Thr Ser
325 330

<210> 10

<211> 2337

<212> DNA

<213> Magnaporthe grisea

<400> 10

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<211> 3235

<212> DNA

<213> Magnaporthe grisea

<400> 11

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taactttccc ctcctaacct atcttcgccc gttcgtctga attcaatcat tttttattgt 180

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agcactccgc agaccttgta cgacaaggtt ctgcaagcac acgtggtcga tgagaagctc 420

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tggagtggca ccaagcagca gcttttgtca actgaccagg tctctttgct tttgtagacc 540

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gccgcgaaac caagccacta tattaacgca acgaaacggg cgctaacatg tttgaatgac 660

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<211> 778

<212> PRT

<213> Magnaporthe grisea

<400> 12

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20 25 30

Ile Asp Arg His Leu Val His Glu Val Thr Ser Pro Gln Ala Phe Glu
35 40 45

Gly Leu Arg Asn Ala Gly Arg Lys Val Arg Arg Pro Asp Cys Thr Leu
50 55 60

Ala Thr Thr Asp His Asn Val Pro Thr Thr Ser Arg Lys Ala Leu Lys
65 70 75 80

Asp Ile Ala Ser Phe Ile Lys Glu Asp Asp Ser Arg Thr Gln Cys Val
85 90 95

Thr Leu Glu Glu Asn Val Lys Glu Phe Gly Val Thr Tyr Phe Gly Leu
100 105 110

Ser Asp Lys Arg Gln Gly Ile Val His Val Ile Gly Pro Glu Gln Gly
115 120 125

Phe Thr Leu Pro Gly Thr Thr Val Val Cys Gly Asp Ser His Thr Ser
130 135 140

Thr His Gly Ala Phe Gly Ala Leu Ala Phe Gly Ile Gly Thr Ser Glu
145 150 155 160

Val Glu His Val Leu Ala Thr Gln Cys Leu Ile Thr Lys Arg Ser Lys
165 170 175

Asn Met Arg Ile Gln Val Asp Gly Glu Leu Ala Pro Gly Val Ser Ser
180 185 190

Lys Asp Val Val Leu His Ala Ile Gly Ile Ile Gly Thr Ala Gly Gly
195 200 205

Thr Gly Ala Val Ile Glu Phe Cys Gly Ser Val Ile Arg Ser Leu Ser
210 215 220

Met Glu Ala Arg Met Ser Ile Cys Asn Met Ser Ile Glu Gly Gly Ala
225 230 235 240

Arg Ala Gly Met Val Ala Pro Asp Glu Ile Thr Phe Glu Tyr Leu Lys
245 250 255

Gly Arg Pro Leu Ala Pro Lys Tyr Asp Ser Pro Glu Trp His Lys Ala
260 265 270

Thr Gln Tyr Trp Lys Asn Leu Gln Ser Asp Pro Gly Ala Lys Tyr Asp
275 280 285

Ile Asp Val Phe Ile Asp Ala Lys Asp Ile Val Pro Thr Leu Thr Trp
290 295 300

Gly Thr Ser Pro Glu Asp Val Val Pro Ile Thr Gly Val Val Pro Asp
305 310 315 320

Pro Glu Thr Phe Ala Thr Glu Ala Lys Lys Ala Asp Gly Arg Arg Met
325 330 335

Leu Gln Tyr Met Gly Leu Lys Ala Gly Thr Pro Met Glu Asp Ile Pro
340 345 350

Val Asp Lys Val Phe Ile Gly Ser Cys Thr Asn Ser Arg Ile Glu Asp
355 360 365

Leu Arg Ala Ala Ala Ala Val Val Lys Gly Arg Lys Lys Ala Pro Asn
370 375 380

Val Lys Ser Ala Met Val Val Pro Gly Ser Gly Leu Val Lys Thr Gln
385 390 395 400

Ala Glu Glu Glu Gly Leu Asp Lys Ile Phe Glu Glu Ala Gly Phe Glu
405 410 415

Trp Arg Glu Ala Gly Cys Ser Met Cys Leu Gly Met Asn Pro Asp Ile
420 425 430

Leu Ala Pro Gln Glu Arg Cys Ala Ser Thr Ser Asn Arg Asn Phe Glu
435 440 445

Gly Arg Gln Gly Ala Gly Gly Arg Thr His Leu Met Ser Pro Val Met
450 455 460

Ala Ala Ala Ala Gly Ile Val Gly Lys Leu Ala Asp Val Arg Lys Leu
465 470 475 480

Thr Asp Tyr Lys Ala Ser Pro His Ile Ala Ala Tyr Gln Lys Ser Thr
485 490 495

Val Thr Lys Pro His Val Asp Glu Arg Ile Asn Gln Asp Ala His Glu
500 505 510

Lys Asp Ile Ile Ala Asp Ile Pro Glu Asp Asn Asn Gly Pro His Thr
515 520 525

Asn Thr Ser Ala Ser Val Gly Thr Ser Ala Gly Leu Pro Lys Phe Thr
530 535 540

Ile Leu Lys Gly Ile Ala Ala Pro Leu Glu Lys Ala Asn Val Asp Thr
545 550 555 560

Asp Ala Ile Ile Pro Lys Gln Phe Leu Lys Thr Ile Lys Arg Thr Gly
565 570 575

Leu Gly Asn Ala Leu Phe Tyr Glu Met Arg Phe Asn Glu Asp Gly Thr
580 585 590

Glu Lys Ser Asp Phe Val Leu Asn Lys Glu Pro Tyr Arg Lys Ala Ser
595 600 605

Ile Leu Val Cys Thr Gly Ala Asn Phe Gly Cys Gly Ser Ser Arg Glu
610 615 620

His Ala Pro Trp Ala Leu Asn Asp Phe Gly Ile Arg Ser Val Ile Ala
625 630 635 640

Pro Ser Phe Ala Asp Ile Phe Phe Asn Asn Ser Phe Lys Asn Gly Met
645 650 655

Leu Pro Ile Pro Ile Lys Asp Gln Ala Gln Ile Glu Ala Ile Ala Ala
660 665 670

Glu Ala Arg Ala Gly Lys Glu Ile Glu Val Asp Leu Pro Asn Gln Leu
675 680 685

Ile Lys Asn Ala Thr Gly Glu Thr Ile Cys Thr Phe Glu Val Glu Glu
690 695 700

Phe Arg Lys His Cys Leu Val Asn Gly Leu Asp Asp Ile Gly Leu Thr
705 710 715 720

Met Gln Met Glu Asp Lys Ile Ala Glu Phe Glu Ala Lys Met Thr Arg
725 730 735

Glu Thr Pro Trp Leu Asp Gly Thr Gly Tyr Leu Lys Arg Lys Gly Gln
740 745 750

Gly Gly Lys Leu Ala Ala Lys Ala Val Pro Val Pro Thr Thr Asn Arg
755 760 765

Gly Glu Glu Lys Lys Glu Pro Leu Glu Trp
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<210> 13

<211> 1650

<212> DNA

<213> Magnaporthe grisea

<400> 13

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<210> 14

<211> 2082

<212> DNA

<213> Magnaporthe grisea

<400> 14
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<210> 15

<211> 549

<212> PRT

<213> Magnaporthe grisea

<400> 15

Met Glu Asn Gly Ala Ala Thr Asn Gly Ala Ser Glu Lys Ser His Ser
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Pro Ser Gln Thr Tyr Leu Ser Thr Arg Gly Asp Asp Tyr Gly Leu Ser
20 25 30

Phe Glu Thr Val Val Leu Lys Gly Leu Ala Ala Asp Gly Gly Leu Phe
35 40 45

Leu Pro Glu Glu Val Pro Ala Ala Thr Glu Trp Gln Ser Trp Lys Asp
50 55 60

Leu Pro Tyr Thr Glu Leu Ala Val Lys Val Leu Ser Leu Tyr Ile Ser
65 70 75 80

Pro Ala Glu Val Pro Thr Glu Asp Leu Arg Ala Leu Val Glu Arg Ser
85 90 95

Tyr Ser Thr Phe Arg Ser Lys Glu Val Val Pro Leu Val Lys Leu Glu
100 105 110

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Asp Asn Leu His Leu Leu Glu Leu Phe His Gly Pro Ser Tyr Ser Phe
        115                 120                 125

Lys Asp Cys Ala Leu Gln Phe Leu Gly Asn Leu Phe Glu Tyr Phe Leu
    130                 135                 140

Thr Arg Lys Asn Lys Gly Lys Glu Gly Lys Asp Arg His His Leu Thr
145                 150                 155                 160

Val Val Gly Ala Thr Ser Gly Asp Thr Gly Ser Ala Ala Ile Tyr Gly
                165                 170                 175

Leu Arg Asn Lys Lys Asp Val Ser Val Phe Ile Leu His Pro Lys Gly
            180                 185                 190

Arg Val Ser Pro Ile Gln Glu Ala Gln Met Thr Thr Val Leu Asp Gln
        195                 200                 205

Asn Val His Asn Leu Ala Val Thr Gly Thr Phe Asp Asp Cys Gln Asp
    210                 215                 220

Ile Val Lys Ala Met Phe Asn Asp Pro Asp Ser Asn Ala Thr Leu Lys
225                 230                 235                 240

Leu Gly Ala Val Asn Ser Ile Asn Trp Ser Arg Ile Leu Ala Gln Ile
                245                 250                 255

Val Tyr Tyr Phe His Ser Tyr Phe Ser Leu Ala Arg Ala Ser Pro Glu
            260                 265                 270

Thr Phe Lys Val Gly Asp Lys Val Arg Phe Val Thr Pro Thr Gly Asn
        275                 280                 285

Phe Gly Asn Ile Leu Ala Gly Tyr Phe Ala Gln Lys Met Gly Leu Pro
    290                 295                 300

Val Asp Lys Leu Val Val Ala Thr Asn Glu Asn Asp Ile Leu Asp Arg
305                 310                 315                 320

Phe Trp Lys Thr Gly Arg Tyr Glu Lys Lys Pro Ala Ser Pro Glu Glu
                325                 330                 335

Ala Ala Gly Gly Leu Pro Gln Asp Gly Val Lys Ala His Glu Glu Gly
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340 345 350

Cys Lys Glu Thr Leu Ser Pro Ala Met Asp Ile Leu Val Ser Ser Asn
355 360 365

Phe Glu Arg Thr Leu Trp Phe Leu Ala Lys Glu Phe Ala Ala Thr Ala
370 375 380

Gly Leu Asn Asp Glu Phe Asn Lys Lys Gln Ala Gly Gln Glu Val Val
385 390 395 400

Ala Trp Tyr Lys Ser Leu Lys Ala Thr Gly Gly Phe Gly Pro Val His
405 410 415

Pro Glu Ile Met Asp Asn Gly Arg Gln Val Phe Glu Ser Glu Arg Val
420 425 430

Ser Asp Thr Gln Thr Leu Glu Met Ile Ala Glu Met Tyr Lys Ala Thr
435 440 445

Lys Tyr Val Leu Asp Pro His Ser Ala Val Gly Val Ala Gly Ala Lys
450 455 460

Arg Ser Met Ser Arg Ala Ser Asn Val Pro His Ile Ala Leu Ser Thr
465 470 475 480

Ala His Pro Ala Lys Phe Ser Gly Ala Val Glu Leu Ala Leu Lys Asp
485 490 495

Gln Lys Glu Phe Asp Phe Thr Lys Gln Val Leu Pro Glu Asp Phe Val
500 505 510

Gly Leu Ala Glu Lys Glu Lys Arg Val Thr Glu Val Ala Ala Asn Trp
515 520 525

Gln Glu Val Arg Glu Ile Val Lys Lys Gln Val Glu Glu Asp Leu Lys
530 535 540

Ala Glu Ser Ser Ala
545

CLAIMS

What is claimed is:

1. A method for identifying a test compound as a candidate for an antibiotic, comprising:
   a) contacting an Asparagine Synthase polypeptide with a test compound; and
   b) detecting the presence or absence of binding between said test compound and said Asparagine Synthase polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

2. The method of claim 1, wherein said Asparagine Synthase polypeptide is a fungal Asparagine Synthase polypeptide.

3. The method of claim 1, wherein said Asparagine Synthase polypeptide is a *Magnaporthe* Asparagine Synthase polypeptide.

4. The method of claim 1, wherein said Asparagine Synthase polypeptide is SEQ ID NO: 3.

5. A method for identifying a test compound as a candidate for an antibiotic, comprising:
   a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal Asparagine Synthase, a polypeptide having at least 50% sequence identity with a fungal Asparagine Synthase, and a polypeptide having at least 10% of the activity of a fungal Asparagine Synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

6. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-aspartate, L-glutamine, and ATP with an Asparagine Synthase;

b) contacting L-aspartate, L-glutamine, and ATP with Asparagine Synthase and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

7. The method of claim 6, wherein said Asparagine Synthase is a fungal Asparagine Synthase.

8. The method of claim 6, wherein said Asparagine Synthase is a *Magnaporthe* Asparagine Synthase.

9. The method of claim 6, wherein said Asparagine Synthase is SEQ ID NO: 3.

10. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with an Asparagine Synthase;

b) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with an Asparagine Synthase and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

11. The method of claim 10, wherein said Asparagine Synthase is a fungal Asparagine Synthase.

12. The method of claim 10, wherein said Asparagine Synthase is a *Magnaporthe* Asparagine Synthase.

13. The method of claim 10, wherein said Asparagine Synthase is SEQ ID NO: 3.

14. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-aspartate, L-glutamine, and ATP with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with Asparagine Synthase, a polypeptide having at least 50% sequence identity with an Asparagine Synthase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an Asparagine Synthase;

b) contacting L-aspartate, L-glutamine, and ATP with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

15. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with an Asparagine Synthase, a polypeptide having at least 50% sequence identity with an Asparagine Synthase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an Asparagine Synthase;

b) contacting L-asparagine, L-glutamate, AMP, and pyrophosphate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-aspartate, L-glutamine, L-asparagine, L-glutamate, ATP, AMP, and/or pyrophosphate, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

16. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of an Asparagine Synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said Asparagine Synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of Asparagine Synthase in steps (a) and (b), wherein a lower expression in the presence of said test compound indicates that said compound is a candidate for an antibiotic.

17. The method of claim 16, wherein said cell, cells, tissue, or organism is, or is derived from a fungus.

18. The method of claim 16, wherein said cell, cells, tissue, or organism is, or is derived from a *Magnaporthe* fungus or fungal cell.

19. The method of claim 16, wherein said Asparagine Synthase is SEQ ID NO: 3.

20. The method of claim 16, wherein the expression of Asparagine Synthase is measured by detecting ASN1 mRNA.

21. The method of claim 16, wherein the expression of Asparagine Synthase is measured by detecting Asparagine Synthase polypeptide.

22. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of an Asparagine Synthase gene, and providing comparison cells having a different form of an Asparagine Synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and comparison cells in the presence of the test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said compound indicates that said compound is a candidate for an antibiotic.

23. The method of claim 22 wherein the cells and the comparison cells are fungal cells.

24. The method of claim 22, wherein the cells and the comparison cells are *Magnaporthe* cells.

25. The method of claim 22, wherein said form and said different form of the Asparagine Synthase are fungal Asparagine Synthases.

26. The method of claim 22, wherein at least one of the forms is a *Magnaporthe* Asparagine Synthase.

27. The method of claim 22, wherein said form and said different form of the Asparagine Synthase are non-fungal Asparagine Synthases.

28. The method of claim 22, wherein one form of the Asparagine Synthase is a fungal Asparagine Synthase, and the different form is a non-fungal Asparagine Synthase.

29. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a gene in the L-asparagine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;

b) contacting said cells and said comparison cells with a said test compound; and

c) determining the growth of said cells and said comparison cells in the presence of said test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

30. The method of claim 29, wherein the cells and the comparison cells are fungal cells.

31. The method of claim 29, wherein the cells and the comparison cells are *Magnaporthe* cells.

32. The method of claim 29, wherein said form and said different form of the L-asparagine biosynthesis gene are fungal L-asparagine biosynthesis genes.

33. The method of claim 29, wherein at least one of the forms is a *Magnaporthe* L-asparagine biosynthesis gene.

34. The method of claim 29, wherein said form and said different form of the L-asparagine biosynthesis genes are non-fungal L-asparagine biosynthesis genes.

35. The method of claim 29, wherein one form of the L-asparagine biosynthesis gene is a fungal L-asparagine biosynthesis gene, and the different form is a non-fungal L-asparagine biosynthesis gene.

36. A method for determining whether the antibiotic candidate of claim 29 has antifungal activity, further comprising: contacting a fungus or fungal cells with said antibiotic candidate and detecting a decrease in growth, viability, or pathogenicity of said fungus or fungal cells, wherein a decrease in growth, viability, or pathogenicity of said fungus or fungal cells indicates that the antibiotic candidate has antifungal activity.

37. A method for identifying a test compound as a candidate for an antibiotic, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-asparagine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism, wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

38. The method of claim 37, wherein said organism is a fungus.

39. The method of claim 37, wherein said organism is *Magnaporthe*.

40. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 3.

41. The nucleic acid of claim 40 comprising the nucleotide sequence of SEQ ID NO: 1.

42. An expression cassette comprising the nucleic acid of claim 40.

43. The isolated nucleic acid of claim 40 comprising a nucleotide sequence with at least 50 to at least 95% sequence identity to SEQ ID NO: 1.

44. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 3.

45. A polypeptide comprising the amino acid sequence of SEQ ID NO: 3.

46. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a 5-Aminolevulinate synthase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said 5-Aminolevulinate synthase polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

47. The method of claim 46, wherein said 5-Aminolevulinate synthase polypeptide is a fungal 5-Aminolevulinate synthase polypeptide.

48. The method of claim 46, wherein said 5-Aminolevulinate synthase polypeptide is a *Magnaporthe* 5-Aminolevulinate synthase polypeptide.

49. The method of claim 46, wherein said 5-Aminolevulinate synthase polypeptide is SEQ ID NO: 6.

50. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal 5-Aminolevulinate synthase, a polypeptide having at least 50% sequence identity with a fungal 5-Aminolevulinate synthase, and a polypeptide having at least 10% of the activity of a fungal 5-Aminolevulinate synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

51. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting succinyl-CoA and glycine with a 5-Aminolevulinate synthase;

b) contacting succinyl-CoA and glycine with 5-Aminolevulinate synthase and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

51. The method of claim 51, wherein said 5-Aminolevulinate synthase is a fungal 5-Aminolevulinate synthase.

52. The method of claim 51, wherein said 5-Aminolevulinate synthase is a *Magnaporthe* 5-Aminolevulinate synthase.

53. The method of claim 51, wherein said 5-Aminolevulinate synthase is SEQ ID NO: 6.

54. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 5-aminolevulinate, CoA, and CO_2 with a 5-Aminolevulinate synthase;

b) contacting 5-aminolevulinate, CoA, and CO_2 with a 5-Aminolevulinate synthase and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

55. The method of claim 54, wherein said 5-Aminolevulinate synthase is a fungal 5-Aminolevulinate synthase.

56. The method of claim 54, wherein said 5-Aminolevulinate synthase is a *Magnaporthe* 5-Aminolevulinate synthase.

57. The method of claim 54, wherein said 5-Aminolevulinate synthase is SEQ ID NO: 6.

58. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting succinyl-CoA and glycine with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with 5-Aminolevulinate synthase; a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase and having at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 5-Aminolevulinate synthase;

b) contacting succinyl-CoA and glycine with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

59. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 5-aminolevulinate, CoA, and CO_2 with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase; a polypeptide having at least 50% sequence identity with a 5-Aminolevulinate synthase and at least 10% of the activity thereof; and a

polypeptide comprising at least 100 consecutive amino acids of a 5-Aminolevulinate synthase;

b) contacting 5-aminolevulinate, CoA, and CO_2, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: succinyl-CoA, glycine, 5-aminolevulinate, CoA, and/or CO_2,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

60. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a 5-Aminolevulinate synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said 5-Aminolevulinate synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of 5-Aminolevulinate synthase in steps (a) and (b),

wherein a lower expression in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

61. The method of claim 60 wherein said cell, cells, tissue, or organism is, or is derived from a fungus.

62. The method of claim 60 wherein said cell, cells, tissue, or organism is, or is derived from a *Magnaporthe* fungus or fungal cell.

63. The method of claim 60, wherein said 5-Aminolevulinate synthase is SEQ ID NO: 6.

64. The method of claim 60, wherein the expression of 5-Aminolevulinate synthase is measured by detecting ALAS1 mRNA.

65. The method of claim 60, wherein the expression of 5-Aminolevulinate synthase is measured by detecting 5-Aminolevulinate synthase polypeptide.

66. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a 5-Aminolevulinate synthase gene, and providing comparison cells having a different form of a 5-Aminolevulinate synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said compound indicates that said compound is a candidate for an antibiotic.

67. The method of claim 66 wherein the cells and the comparison cells are fungal cells.

68. The method of claim 66 wherein the cells and the comparison cells are *Magnaporthe* cells.

69. The method of claim 66 wherein said form and said comparison form of the 5-Aminolevulinate synthase are fungal 5-Aminolevulinate synthases.

70. The method of claim 66, wherein at least one of the forms is a *Magnaporthe* 5-Aminolevulinate synthase.

71. The method of claim 66 wherein said form and said comparison form of the 5-Aminolevulinate synthase are non-fungal 5-Aminolevulinate synthases.

72. The method of claim 66 wherein one form of the 5-Aminolevulinate synthase is a fungal 5-Aminolevulinate synthase, and the other form is a non-fungal 5-Aminolevulinate synthase.

73. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a gene in the heme biochemical and/or genetic pathway and providing comparison cells having a different form of said gene.

b) contacting said cells and said comparison cells with a test compound,

c) determining the growth of said cells and said comparison cells in the presence of said test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

74. The method of claim 73 wherein the cells and the comparison cells are fungal cells.

75. The method of claim 73 wherein the cells and the comparison cells are *Magnaporthe* cells.

76. The method of claim 73 wherein said form and said different form of the heme biosynthesis gene are fungal heme biosynthesis genes.

77. The method of claim 73, wherein at least one form is a *Magnaporthe* heme biosynthesis gene.

78. The method of claim 73 wherein said form and said different form of the heme biosynthesis genes are non-fungal heme biosynthesis genes.

79. The method of claim73 wherein one form of the heme biosynthesis gene is a fungal heme biosynthesis gene, and the different form is a non-fungal heme biosynthesis gene.

80. A method for identifying a test compound as a candidate for an antibiotic, comprising:

(a) providing paired growth media; comprising a first medium and a second medium, wherein said second medium contains a higher level of 5-aminolevulinate than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism,

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

81. The method of claim 80, wherein said organism is a fungus.

82. The method of claim 80, wherein said organism is *Magnaporthe*.

83. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 6.

84. The nucleic acid of claim 83 comprising the nucleotide sequence of SEQ ID NO: 4.

85. An expression cassette comprising the nucleic acid of claim 83.

86. The isolated nucleic acid of claim 83 comprising a nucleotide sequence with at least 50 to at least 95% sequence identity to SEQ ID NO: 4.

87. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 6.

88. A polypeptide comprising the amino acid sequence of SEQ ID NO: 6.

89. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a histidinol-phosphatase polypeptide with a test compound; and

b) detecting the presence or absence of binding between said test compound and said histidinol-phosphatase polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

90. . The method of claim 89, wherein said histidinol-phosphatase polypeptide is a fungal histidinol-phosphatase polypeptide.

91. The method of claim 89, wherein said histidinol-phosphatase polypeptide is a *Magnaporthe* histidinol-phosphatase polypeptide.

92. The method of claim 89, wherein said histidinol-phosphatase polypeptide is SEQ ID NO: 9.

93. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal histidinol-phosphatase; a polypeptide having at least 50% sequence identity with; and a polypeptide having at least 10% of the activity of a fungal histidinol-phosphatase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

94. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol phosphate and H_2O with a histidinol-phosphatase;

b) contacting L-histidinol phosphate and H_2O with histidinol-phosphatase and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

95. The method of claim 94, wherein said histidinol-phosphatase is a fungal histidinol-phosphatase.

96. The method of claim 94, wherein said histidinol-phosphatase is a *Magnaporthe* histidinol-phosphatase.

97. The method of claim 94, wherein said histidinol-phosphatase is SEQ ID NO: 9.

98. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol and orthophosphate with a histidinol-phosphatase;

b) contacting L-histidinol and orthophosphate with a histidinol-phosphatase and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

99. The method of claim 98, wherein said histidinol-phosphatase is a fungal histidinol-phosphatase.

100. The method of claim 98, wherein said histidinol-phosphatase is a *Magnaporthe* histidinol-phosphatase.

101. The method of claim 98, wherein said histidinol-phosphatase is SEQ ID NO: 9.

102. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol phosphate and H_2O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with histidinol-phosphatase; a polypeptide having at least 50% sequence identity with a histidinol-phosphatase and having at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a histidinol-phosphatase;

b) contacting L-histidinol phosphate and H_2O with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

103. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-histidinol and orthophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a histidinol-phosphatase; a polypeptide having at least 50% sequence identity with a histidinol-phosphatase and at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a histidinol-phosphatase;

b) contacting L-histidinol and orthophosphate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: L-histidinol phosphate, H_2O, L-histidinol, and/or orthophosphate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

104. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a histidinol-phosphatase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said histidinol-phosphatase in said cell, cells, tissue, or organism; and

c) comparing the expression of histidinol-phosphatase in steps (a) and (b),

wherein a lower expression in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

105. The method of claim 104 wherein said cell, cells, tissue, or organism is, or is derived from a fungus.

106. The method of claim 104 wherein said cell, cells, tissue, or organism is, or is derived from a *Magnaporthe* fungus or fungal cell.

107. The method of claim 104, wherein said histidinol-phosphatase is SEQ ID NO: 9.

108. The method of claim 104, wherein the expression of histidinol-phosphatase is measured by detecting HISP1 mRNA.

109. The method of claim 104, wherein the expression of histidinol-phosphatase is measured by detecting histidinol-phosphatase polypeptide.

110. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a histidinol-phosphatase gene, and providing comparison cells having a different form of a histidinol-phosphatase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said compound indicates that said compound is a candidate for an antibiotic.

111. The method of claim 110 wherein the cells and the comparison cells are fungal cells.

112. The method of claim 110 wherein the cells and the comparison cells are *Magnaporthe* cells.

113. The method of claim 110 wherein said form and said comparison form of the histidinol-phosphatase are fungal histidinol-phosphatases.

114. The method of claim 110, wherein at least one of the forms is a *Magnaporthe* histidinol-phosphatase.

115. The method of claim 110 wherein said form and said comparison form of the histidinol-phosphatase are non-fungal histidinol-phosphatases.

116. The method of claim 110 wherein one form of the histidinol-phosphatase is a fungal histidinol-phosphatase, and the other form is a non-fungal histidinol-phosphatase.

117. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a gene in the L-histidine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene.

b) contacting said cells and said comparison cells with a test compound,

c) determining the growth of said cells and said comparison cells in the presence of said test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

118. The method of claim 117 wherein the cells and the comparison cells are fungal cells.

119. The method of claim 117 wherein the cells and the comparison cells are *Magnaporthe* cells.

120. The method of claim 117 wherein said form and said different form of the L-histidine biosynthesis gene are fungal L-histidine biosynthesis genes.

121. The method of claim 117, wherein at least one form is a *Magnaporthe* L-histidine biosynthesis gene.

122. The method of claim 117 wherein said form and said different form of the L-histidine biosynthesis genes are non-fungal L-histidine biosynthesis genes.

123. The method of claim 117 wherein one form of the L-histidine biosynthesis gene is a fungal L-histidine biosynthesis gene, and the different form is a non-fungal L-histidine biosynthesis gene.

124. A method for identifying a test compound as a candidate for an antibiotic, comprising:

(a) providing paired growth media; comprising a first medium and a second medium, wherein said second medium contains a higher level of L-histidine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism,

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

125. The method of claim 124, wherein said organism is a fungus.

126. The method of claim 124, wherein said organism is *Magnaporthe.*

127. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 9.

128. The nucleic acid of claim 127 comprising the nucleotide sequence of SEQ ID NO:

129. An expression cassette comprising the nucleic acid of claim 128.

130. The isolated nucleic acid of claim 127 comprising a nucleotide sequence with at least 50 to at least 95% sequence identity to SEQ ID NO: 7.

131. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 9

132. A polypeptide comprising the amino acid sequence of SEQ ID NO: 9.

133. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a 3-Isopropylmalate dehydratase polypeptide with said test compound; and

b) detecting the presence or absence of binding between a test compound and said 3-Isopropylmalate dehydratase polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

134. The method of claim 133, wherein said 3-Isopropylmalate dehydratase polypeptide is a fungal 3-Isopropylmalate dehydratase polypeptide.

135. The method of claim 133, wherein said 3-Isopropylmalate dehydratase polypeptide is a *Magnaporthe* 3-Isopropylmalate dehydratase polypeptide.

136. The method of claim 133, wherein said 3-Isopropylmalate dehydratase polypeptide is SEQ ID NO: 12.

137. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a fungal 3-Isopropylmalate dehydratase; and a polypeptide having at least 10% of the activity of a fungal 3-Isopropylmalate dehydratase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide,

wherein binding indicates that said test compound is a candidate for an antibiotic.

138. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 2-Isopropylmalate and H_2O with a 3-Isopropylmalate dehydratase;

b) contacting 2-Isopropylmalate and H_2O with 3-Isopropylmalate dehydratase and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

139. The method of claim 138, wherein said 3-Isopropylmalate dehydratase is a fungal 3-Isopropylmalate dehydratase.

140. The method of claim 138, wherein said 3-Isopropylmalate dehydratase is a *Magnaporthe* 3-Isopropylmalate dehydratase.

141. The method of claim 138, wherein said 3-Isopropylmalate dehydratase is SEQ ID NO: 12.

142. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 3-Isopropylmalate with a 3-Isopropylmalate dehydratase;

b) contacting 3-Isopropylmalate with a 3-Isopropylmalate dehydratase and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

143. The method of claim 142, wherein said 3-Isopropylmalate dehydratase is a fungal 3-Isopropylmalate dehydratase.

144. The method of claim 142, wherein said 3-Isopropylmalate dehydratase is a *Magnaporthe* 3-Isopropylmalate dehydratase.

145. The method of claim 142, wherein said 3-Isopropylmalate dehydratase is SEQ ID NO: 12.

146. A method for determining whether the antibiotic candidate of claim 142 has antifungal activity, further comprising:

contacting a fungus or fungal cells with said antibiotic candidate and detecting a decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

147. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 2-Isopropylmalate and H_2O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase and having at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 3-Isopropylmalate dehydratase;

b) contacting 2-Isopropylmalate and H_2O with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

148. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting 3-Isopropylmalate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase; a polypeptide having at least 50% sequence identity with a 3-Isopropylmalate dehydratase and at least 10% of the activity thereof; and a polypeptide comprising at least 100 consecutive amino acids of a 3-Isopropylmalate dehydratase;

b) contacting 3-Isopropylmalate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: 2-Isopropylmalate, H_2O, and/or 3-Isopropylmalate,

wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

149. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a 3-Isopropylmalate dehydratase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said 3-Isopropylmalate dehydratase in said cell, cells, tissue, or organism; and

c) comparing the expression of 3-Isopropylmalate dehydratase in steps (a) and (b), wherein a lower expression in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

150. The method of claim 149 wherein said cell, cells, tissue, or organism is, or is derived from a fungus.

151. The method of claim 149 wherein said cell, cells, tissue, or organism is, or is derived from a *Magnaporthe* fungus or fungal cell.

152. The method of claim 149, wherein said 3-Isopropylmalate dehydratase is SEQ ID NO: 12.

153. The method of claim 149, wherein the expression of 3-Isopropylmalate dehydratase is measured by detecting IPMD1 mRNA.

154. The method of claim 149, wherein the expression of 3-Isopropylmalate dehydratase is measured by detecting 3-Isopropylmalate dehydratase polypeptide.

155. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a 3-Isopropylmalate dehydratase gene, and providing comparison cells having a different form of a 3-Isopropylmalate dehydratase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and comparison cells in the presence of the test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said compound indicates that said compound is a candidate for an antibiotic.

156. The method of claim 155 wherein the cells and the comparison cells are fungal cells.

157. The method of claim 155 wherein the cells and the comparison cells are *Magnaporthe* cells.

158. The method of claim 155 wherein said form and said comparison form of the 3-Isopropylmalate dehydratase are fungal 3-Isopropylmalate dehydratases.

159. The method of claim 155, wherein at least one of the forms is a *Magnaporthe* 3-Isopropylmalate dehydratase.

160. The method of claim 155 wherein said form and said comparison form of the 3-Isopropylmalate dehydratase are non-fungal 3-Isopropylmalate dehydratases.

161. The method of claim 155 wherein one form of the 3-Isopropylmalate dehydratase is a fungal 3-Isopropylmalate dehydratase, and the other form is a non-fungal 3-Isopropylmalate dehydratase.

162. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a gene in the L-leucine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene.

b) contacting said cells and said comparison cells with a said test compound,

c) determining the growth of said cells and said comparison cells in the presence of said test compound,

wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

163. The method of claim 162 wherein the cells and the comparison cells are fungal cells.

164. The method of claim 162 wherein the cells and the comparison cells are *Magnaporthe* cells.

165. The method of claim 162 wherein said form and said different form of the L-leucine biosynthesis gene are fungal L-leucine biosynthesis genes.

166. The method of claim 162, wherein at least one form is a *Magnaporthe* L-leucine biosynthesis gene.

167. The method of claim 162 wherein said form and said different form of the L-leucine biosynthesis genes are non-fungal L-leucine biosynthesis genes.

168. The method of claim 162 wherein one form of the L-leucine biosynthesis gene is a fungal L-leucine biosynthesis gene, and the different form is a non-fungal L-leucine biosynthesis gene.

169. A method for identifying a test compound as a candidate for an antibiotic, comprising:

(a) providing paired growth media; comprising a first medium and a second medium, wherein said second medium contains a higher level of L-leucine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism,

wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

170. The method of claim 169, wherein said organism is a fungus.

171. The method of claim 169, wherein said organism is *Magnaporthe*.

172. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 12.

173. The nucleic acid of claim 172 comprising the nucleotide sequence of SEQ ID NO: 10.

174. An expression cassette comprising the nucleic acid of claim 173.

175. The isolated nucleic acid of claim 172 comprising a nucleotide sequence with at least 50 to at least 95% sequence identity to SEQ ID NO: 10.

176. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 12

177. A polypeptide comprising the amino acid sequence of SEQ ID NO: 12.

178. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a Threonine synthase polypeptide with said test compound; and

b) detecting the presence or absence of binding between a test compound and said Threonine synthase polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

179. The method of claim 178, wherein said Threonine synthase polypeptide is a fungal Threonine synthase polypeptide.

180. The method of claim 178, wherein said Threonine synthase polypeptide is a *Magnaporthe* Threonine synthase polypeptide.

181. The method of claim 178, wherein said Threonine synthase polypeptide is SEQ ID NO: 15.

182. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting a test compound with at least one polypeptide selected from the group consisting of: a polypeptide having at least ten consecutive amino acids of a fungal Threonine synthase, and a polypeptide having at least 50% sequence identity with a fungal Threonine synthase, and a polypeptide having at least 10% of the activity of a fungal Threonine synthase; and

b) detecting the presence and/or absence of binding between said test compound and said polypeptide, wherein binding indicates that said test compound is a candidate for an antibiotic.

183. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting O-phospho-L-homoserine and water with a Threonine synthase;

b) contacting O-phospho-L-homoserine and water with Threonine synthase and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

184. The method of claim 183, wherein said Threonine synthase is a fungal Threonine synthase.

185. The method of claim 183, wherein said Threonine synthase is a *Magnaporthe* Threonine synthase.

186.The method of claim 183, wherein said Threonine synthase is SEQ ID NO: 15.

187. A method for determining whether the antibiotic candidate of claim 8 has antifungal activity, further comprising contacting a fungus or fungal cells with said antibiotic candidate and detecting a decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

188. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-threonine and orthophosphate with a Threonine synthase;

b) contacting L-threonine and orthophosphate with a Threonine synthase and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

189. The method of claim 188, wherein said Threonine synthase is a fungal Threonine synthase.

190. The method of claim 188, wherein said Threonine synthase is a *Magnaporthe* Threonine synthase.

191. The method of claim 188, wherein said Threonine synthase is SEQ ID NO: 15.

192. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting O-phospho-L-homoserine and water with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with Threonine synthase, and a polypeptide having at least 50% sequence identity with a

Threonine synthase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a Threonine synthase;

b) contacting O-phospho-L-homoserine and water with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

193. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) contacting L-threonine and orthophosphate with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with a Threonine synthase, and a polypeptide having at least 50% sequence identity with a Threonine synthase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a Threonine synthase;

b) contacting L-threonine and orthophosphate, with said polypeptide and a test compound; and

c) determining the change in concentration for at least one of the following: O-phospho-L-homoserine, L-threonine, orthophosphate, and water, wherein a change in concentration for any of the above substances between steps (a) and (b) indicates that said test compound is a candidate for an antibiotic.

194. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) measuring the expression of a Threonine synthase in a cell, cells, tissue, or an organism in the absence of a test compound;

b) contacting said cell, cells, tissue, or organism with said test compound and measuring the expression of said Threonine synthase in said cell, cells, tissue, or organism; and

c) comparing the expression of Threonine synthase in steps (a) and (b), wherein a lower expression in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

195. The method of claim 194, wherein said cell, cells, tissue, or organism is, or is derived from a fungus.

196. The method of claim 194, wherein said cell, cells, tissue, or organism is, or is derived from a *Magnaporthe* fungus or fungal cell.

197. The method of claim 194, wherein said Threonine synthase is SEQ ID NO: 15.

198. The method of claim 194, wherein the expression of Threonine synthase is measured by detecting THR4 mRNA.

199. The method of claim 194, wherein the expression of Threonine synthase is measured by detecting Threonine synthase polypeptide.

200. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a Threonine synthase gene, and providing comparison cells having a different form of a Threonine synthase gene; and

b) contacting said cells and said comparison cells with a test compound and determining the growth of said cells and comparison cells in the presence of the test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said compound indicates that said compound is a candidate for an antibiotic.

201. The method of claim 200 wherein the cells and the comparison cells are fungal cells.

202. The method of claim 200 wherein the cells and the comparison cells are *Magnaporthe* cells.

203. The method of claim 200 wherein said form and said different form of the Threonine synthase are fungal Threonine synthases.

204. The method of claim 200, wherein at least one of the forms is a *Magnaporthe* Threonine synthase.

205. The method of claim 200, wherein said form and said different form of the Threonine synthase are non-fungal Threonine synthases.

206. The method of claim 200, wherein one form of the Threonine synthase is a fungal Threonine synthase, and the different form is a non-fungal Threonine synthase.

207. A method for identifying a test compound as a candidate for an antibiotic, comprising:

a) providing cells having one form of a gene in the L-threonine biochemical and/or genetic pathway and providing comparison cells having a different form of said gene;

b) contacting said cells and said comparison cells with a said test compound; and

c) determining the growth of said cells and said comparison cells in the presence of said test compound, wherein a difference in growth between said cells and said comparison cells in the presence of said test compound indicates that said test compound is a candidate for an antibiotic.

208. The method of claim 207, wherein the cells and the comparison cells are fungal cells.

209. The method of claim 207, wherein the cells and the comparison cells are *Magnaporthe* cells.

210. The method of claim 207, wherein said form and said different form of the L-threonine biosynthesis gene are fungal L-threonine biosynthesis genes.

211. The method of claim 207, wherein at least one of the forms is a *Magnaporthe* L-threonine biosynthesis gene.

212. The method of claim 207, wherein said form and said different form of the L-threonine biosynthesis genes are non-fungal L-threonine biosynthesis genes.

213. The method of claim 207, wherein one form of the L-threonine biosynthesis gene is a fungal L-threonine biosynthesis gene, and the different form is a non-fungal L-threonine biosynthesis gene.

214. A method for identifying a test compound as a candidate for an antibiotic, comprising:

(a) providing paired growth media comprising a first medium and a second medium, wherein said second medium contains a higher level of L-threonine than said first medium;

(b) contacting an organism with a test compound;

(c) inoculating said first and said second media with said organism; and

(d) determining the growth of said organism, wherein a difference in growth of the organism between said first and said second media indicates that said test compound is a candidate for an antibiotic.

215. The method of claim 214, wherein said organism is a fungus.

216. The method of claim 214, wherein said organism is *Magnaporthe*.

217. An isolated nucleic acid comprising a nucleotide sequence that encodes a polypeptide of SEQ ID NO: 15.

218. The nucleic acid of claim 217 comprising the nucleotide sequence of SEQ ID NO: 13.

219. An expression cassette comprising the nucleic acid of claim 218.

220. The isolated nucleic acid of claim 217 comprising a nucleotide sequence with at least 50 to at least 95% sequence identity to SEQ ID NO: 13.

221. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 15.

222. A polypeptide comprising the amino acid sequence of SEQ ID NO: 15.

Figure 1

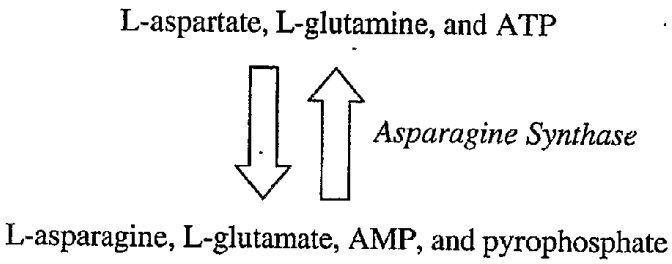

Figure 2

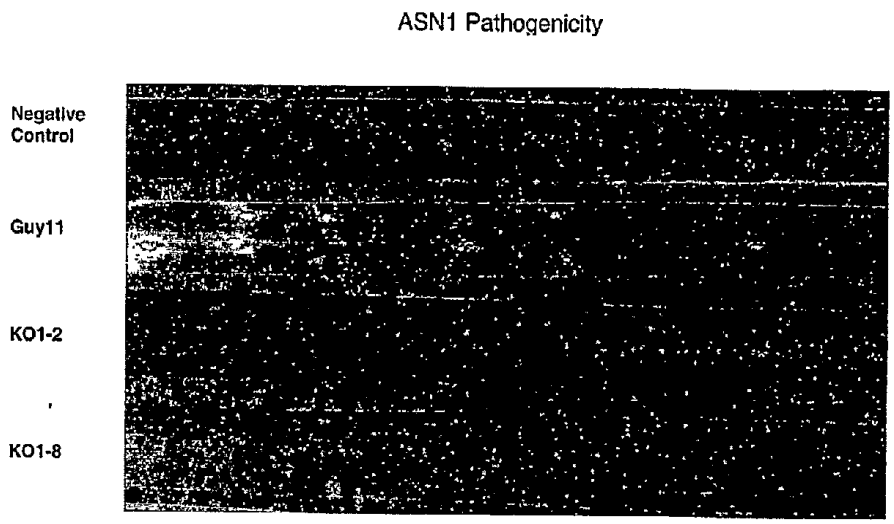

Figure 3A

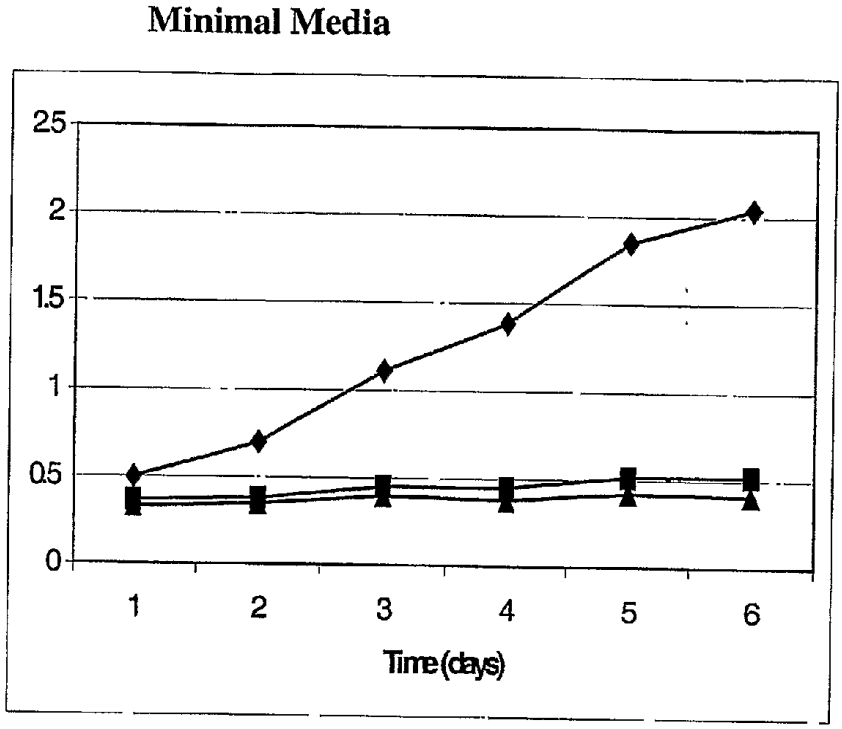

Figure 3B

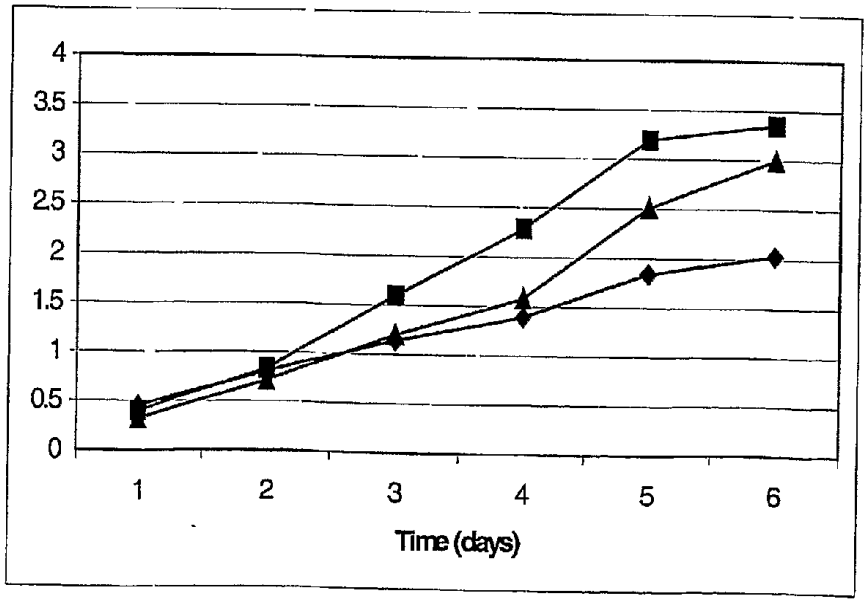

Figure 4.

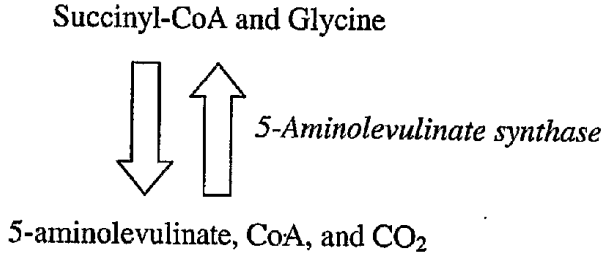

Figure 5.

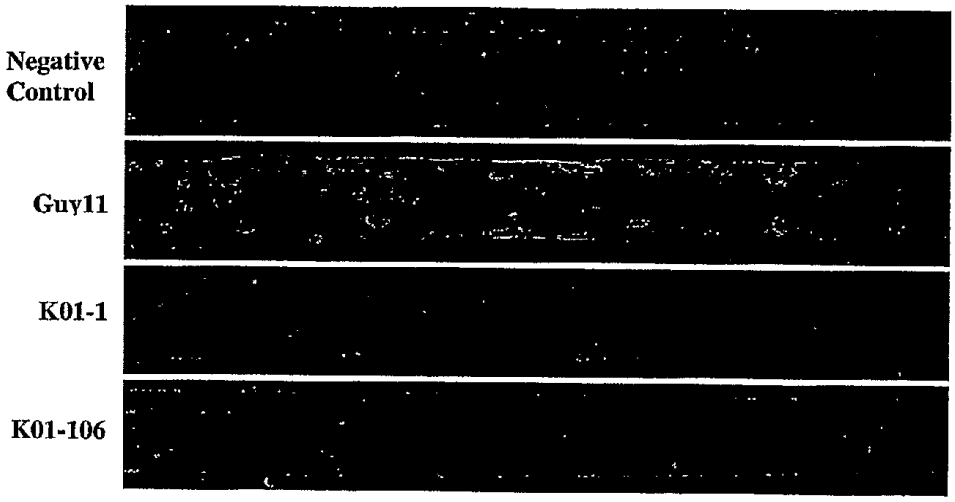

Figure 6A. **Minimal Media**

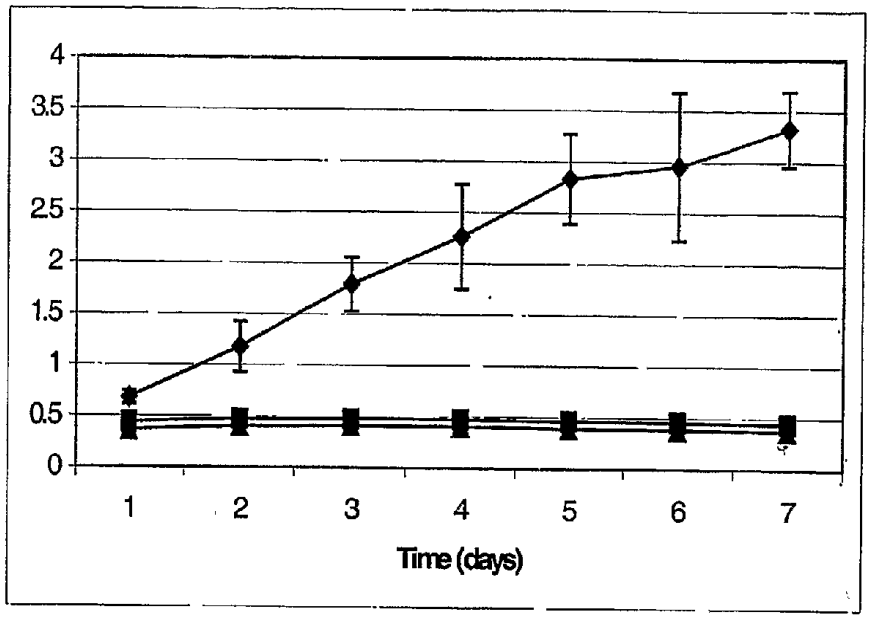

Figure 6B **Minimal Media + d5-aminolevulinic acid**

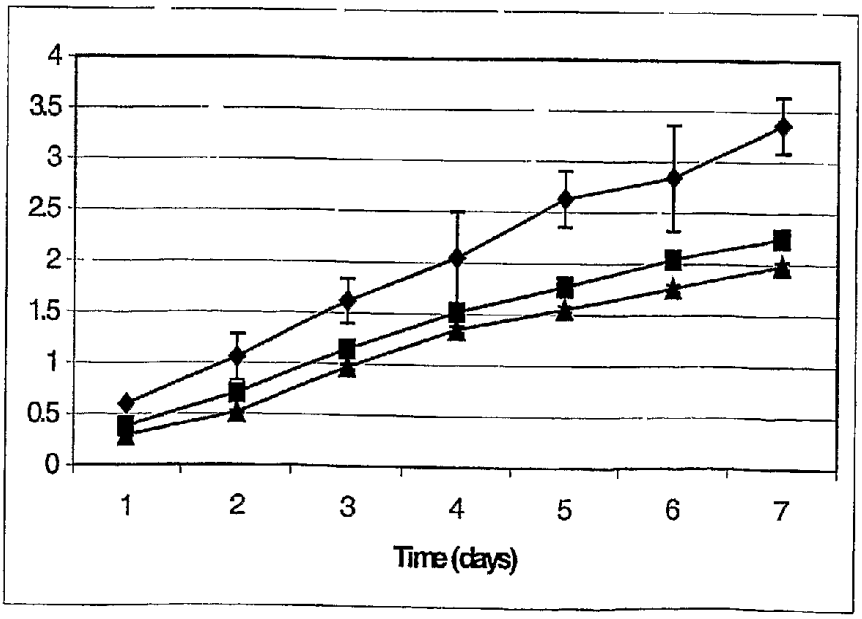

Figure 7

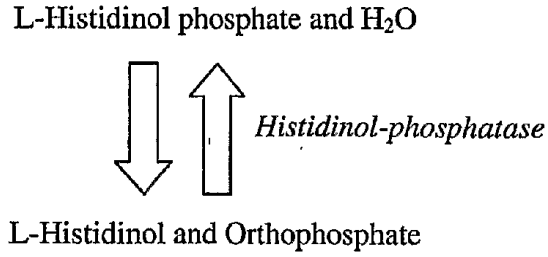

Figure 8

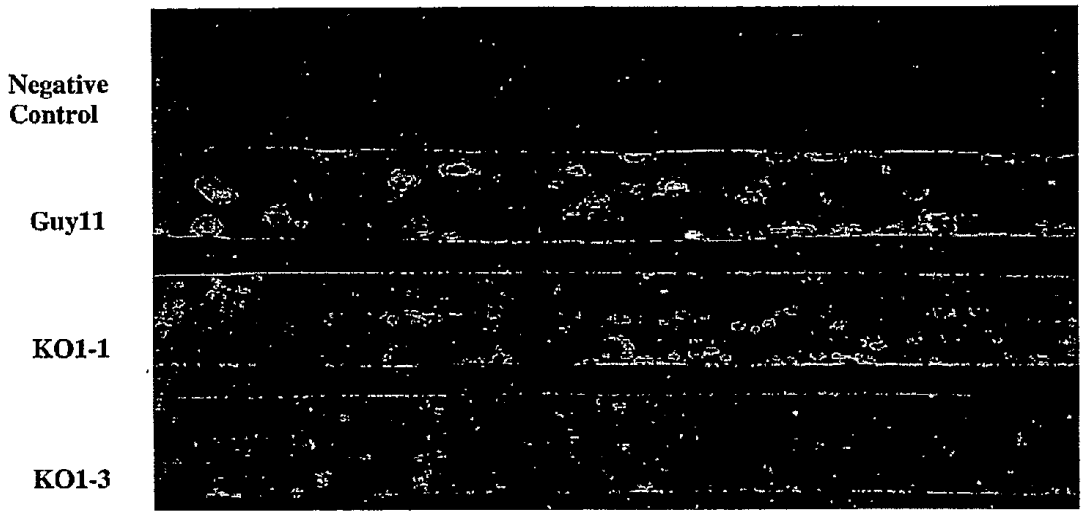

Figure 9A

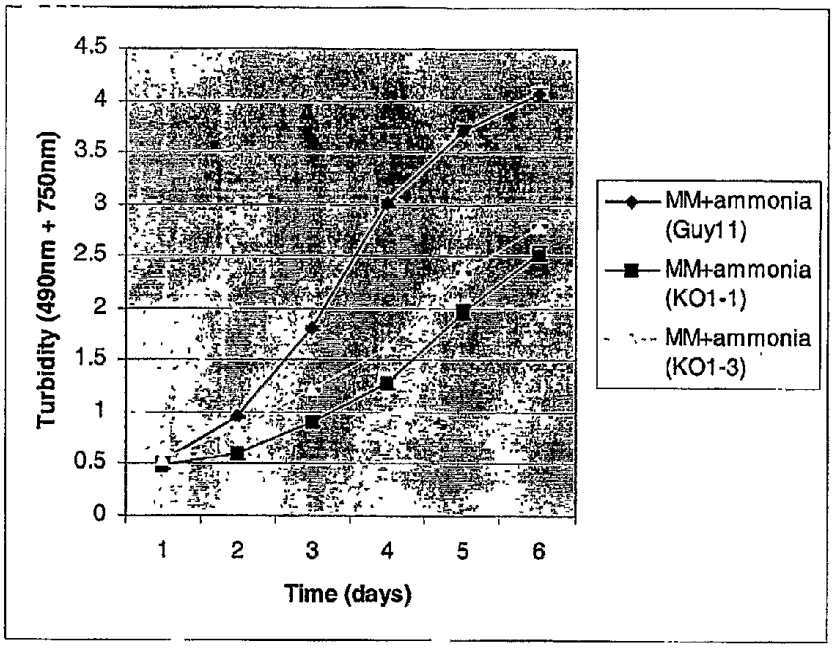

Figure 9B

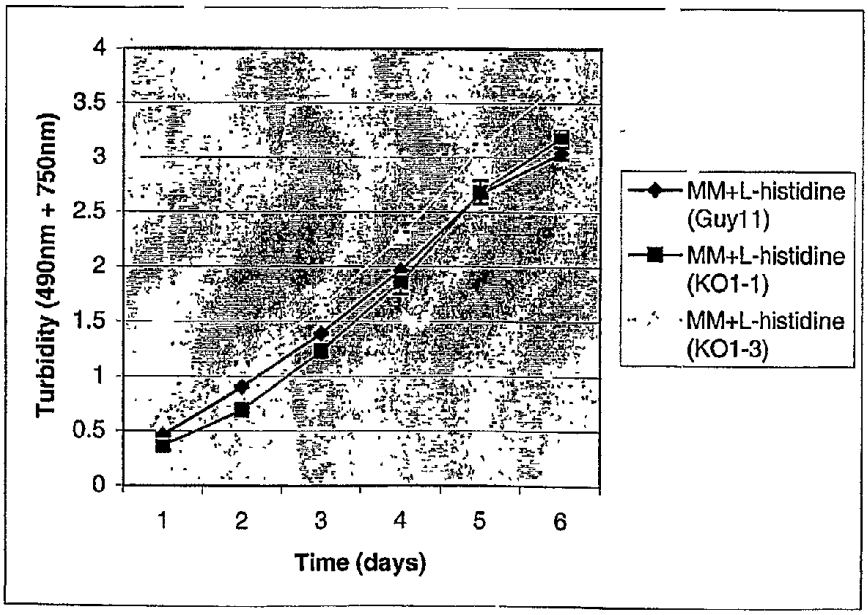

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Figure 10

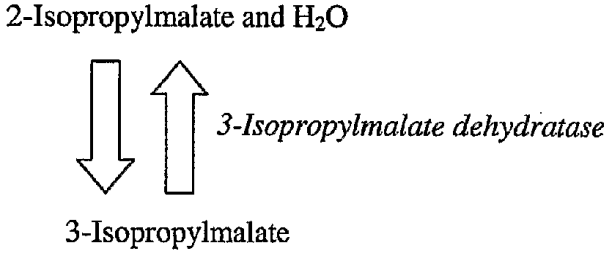


Figure 11

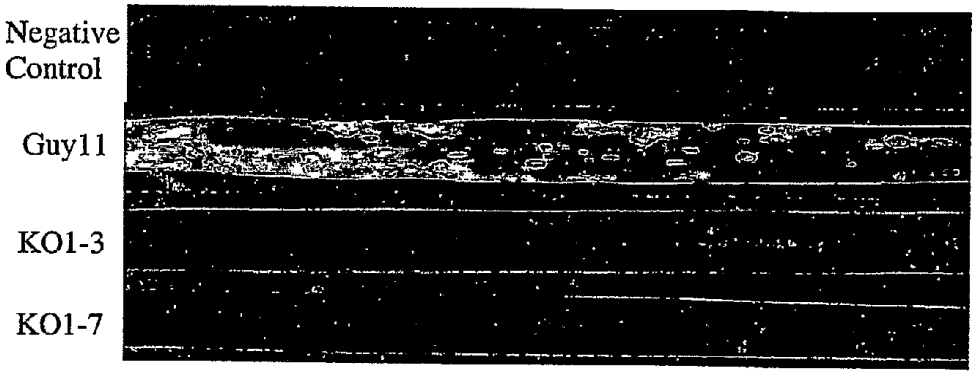


Figure 12A

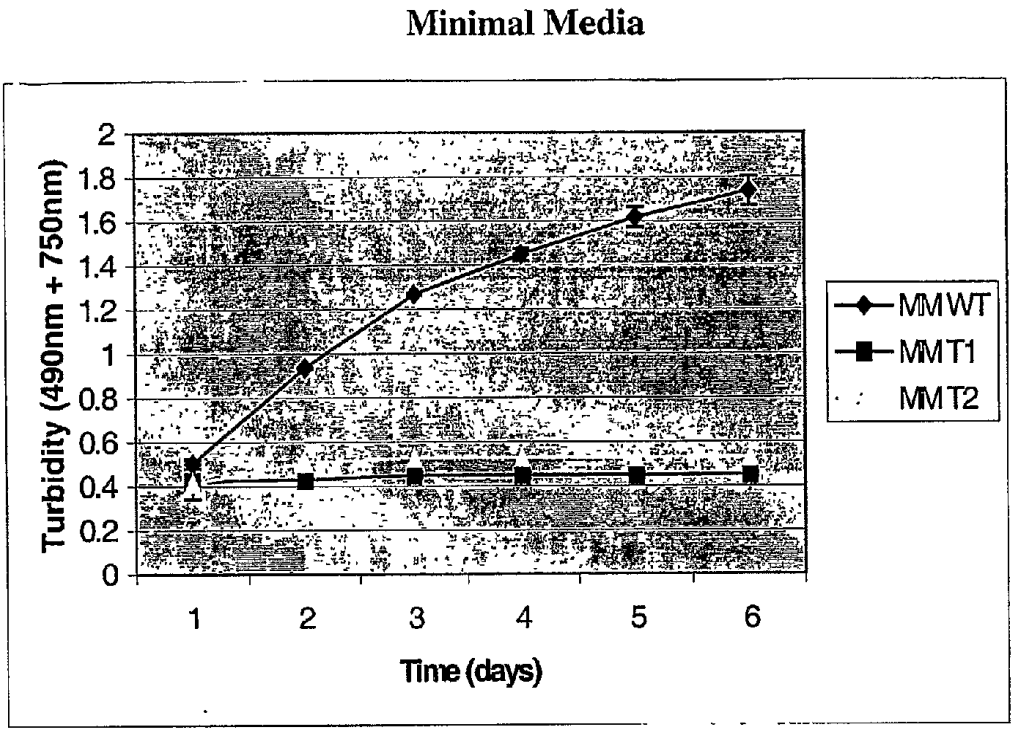


Figure 12B

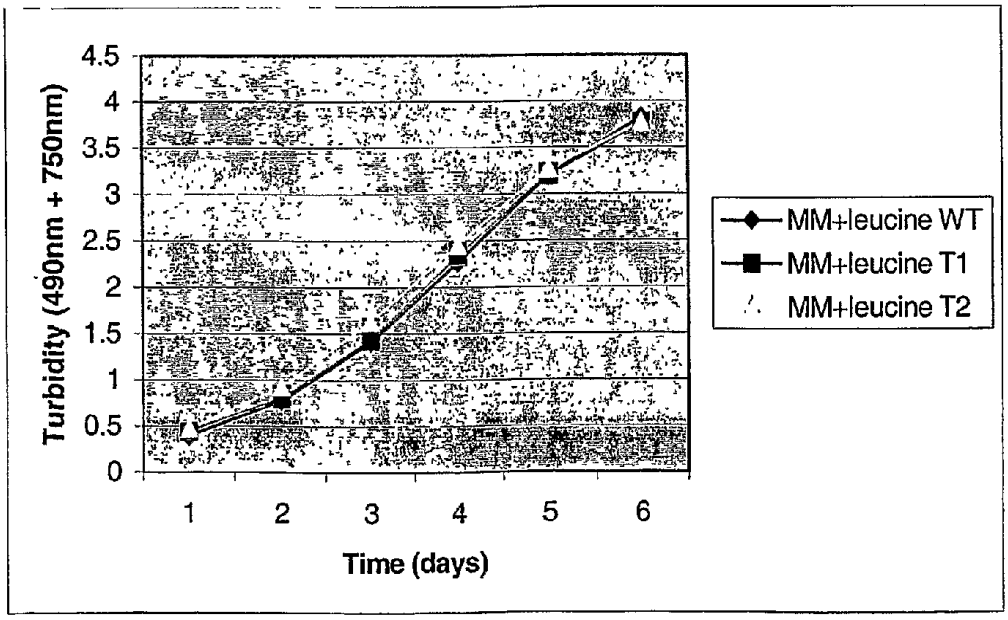


Figure 13

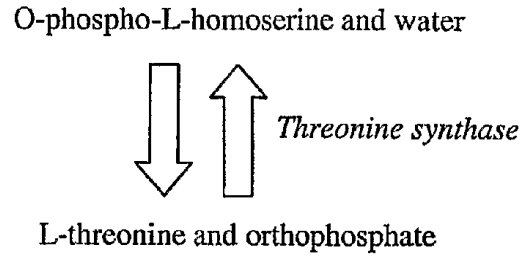

O-phospho-L-homoserine and water
Threonine synthase
L-threonine and orthophosphate

Figure 14

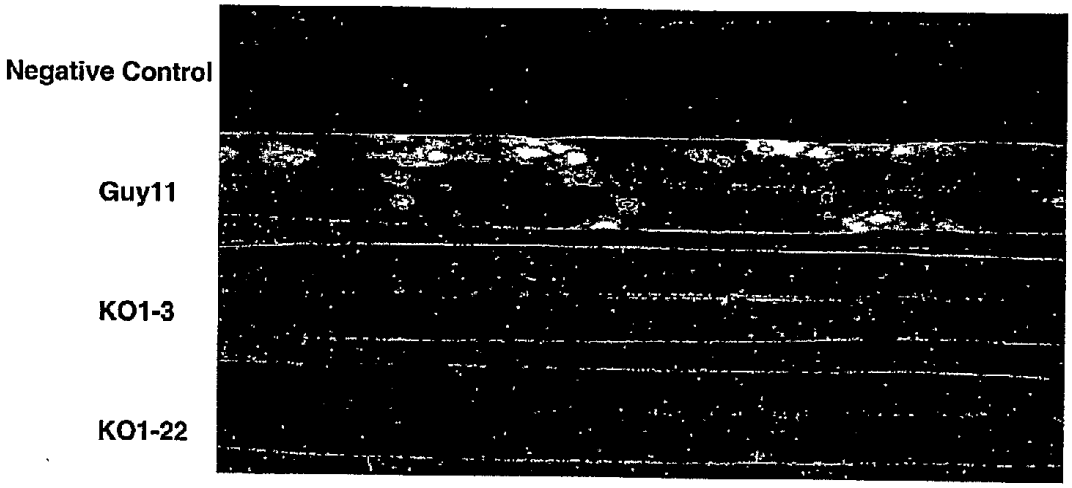


Figure 15A

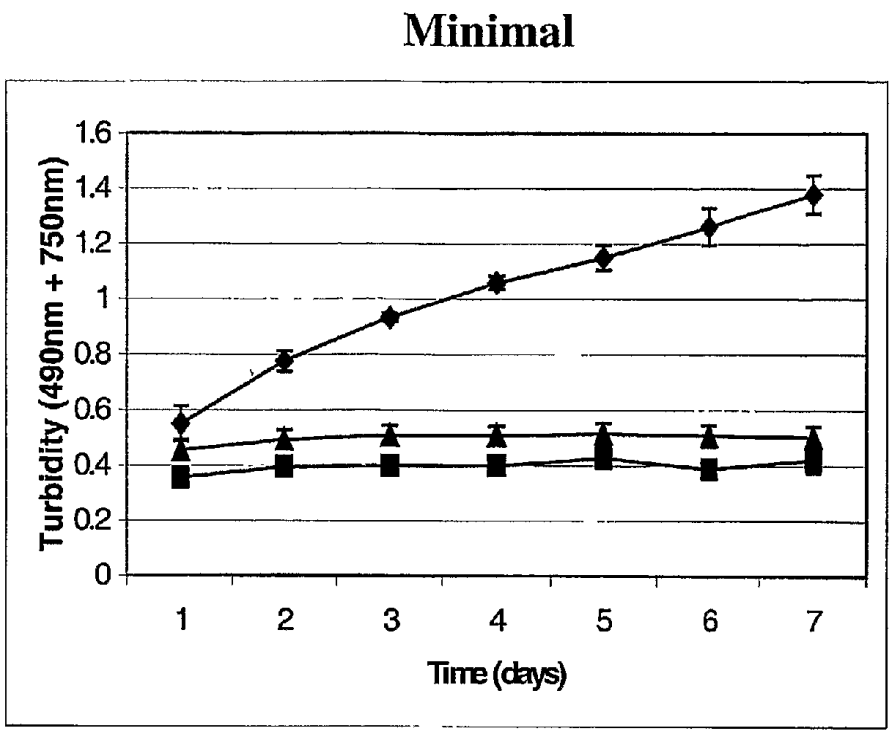
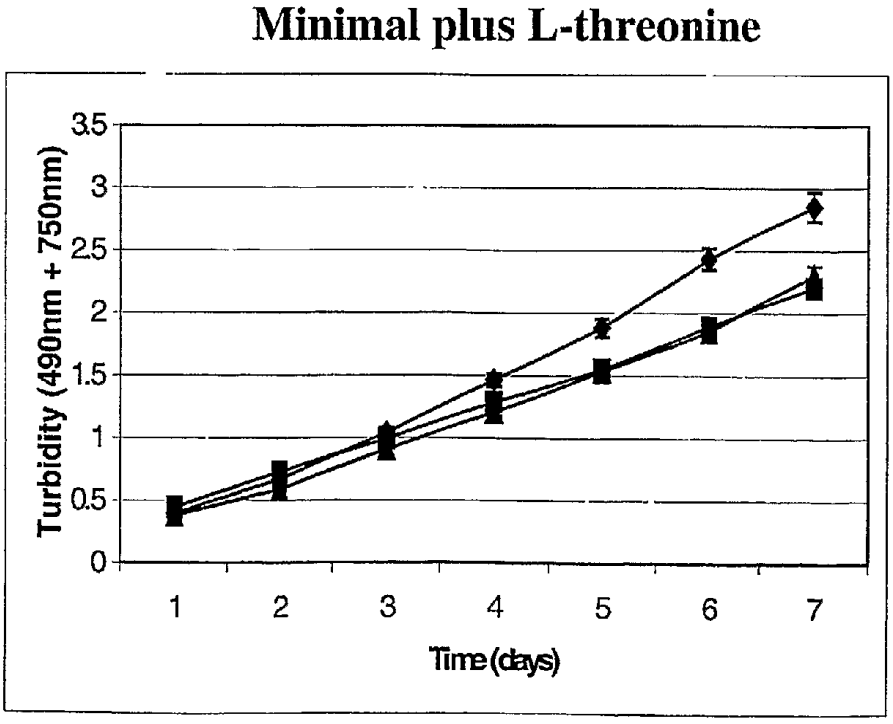


Figure 15B



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