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(71) **Applicant** *(for all designated States except US)*: **PARA-DIGM GENETICS, INC.** [US/US]; 108 Alexander Drive, P.O. Box 14528, Research Triangle Park, NC 27709-4528 (US).

(72) **Inventors; and**

(75) **Inventors/Applicants** *(for US only)*: **TANZER, Matthew** [US/US]; 6 Drakesway Court, Durham, NC 27713 (US). **SHUSTER, Jeffrey** [US/US]; 110 Graylyn Drive, Chapel Hill, NC 27516 (US). **HAMER, Lisbeth** [DK/US]; 3414 Dover Avenue, Durham, NC 27707 (US). **ADACHI, Kiichi** [JP/US]; 31 Dansey Circle Ln, Durham, NC 27713 (US). **DEZWAAN, Todd** [US/US]; 2216 Colony Woods Dr, Apex, NC 27502 (US). **LO, Sze-Chung** [CN/CN]; 2308 LeeHang House, Shun Lee Estate, Hong Kong (CN). **MONTENEGRO-CHAMORRO, Maria, Victoria**

[CO/US]; 2324 Winter Walk Cir, Morrisville, NC 27560 (US). **FRANK, Sheryl** [US/US]; 600 Mahone Street, #11, Durham, NC 27713 (US). **DARVEAUX, Blaise** [US/US]; 104 Bonparte Drive, Hillsborough, NC 27278 (US). **MA-HANTY, Sanjoy** [IN/US]; 514 Highgrove Drive, Chapel Hill, NC 27516 (US). **HEINIGER, Ryan** [US/US]; 1901 Trailwood Hgts, Apt. 101, Raleigh, NC 27603 (US). **SKALCHUNES, Amy** [US/US]; 3505 Apache Drive, Raleigh, NC 27609 (US). **PAN, Huaquin** [CN/US]; 2203 Henniker Street, Apex, NC 27502 (US). **TARPEY, Rex** [US/US]; 1911 Housatonic Lane, Apex, NC 27502 (US).

(74) **Agent: SPENCER, Deborah**; Paradigm Genetics, Inc., 108 Alexander Drive, P.O. Box 14528, Research Triangle Park, NC 27709-4528 (US).

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[Continued on next page]

(54) **Title:** METHODS FOR THE IDENTIFICATION OF INHIBITORS OF ALPHA-AMINOADIPATE REDUCTASE AND HOMOCITRATE SYNTHASE AS ANTIBIOTICS

L-2-Aminoadipate + NADPH + ATP
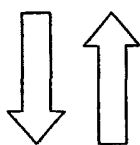
α-Aminoadipate Reductase + Mg^{2+}

L-2-Aminoadipate 6-semialdehyde + NADP+ + AMP + pyrophosphate + H_2O

(57) **Abstract:** The present inventors have discovered that α-Aminoadipate Reductase and Homocitrate Synthase are each essential for fungal pathogenicity. Specifically, the inhibition of α-Aminoadipate Reductase or Homocitrate Synthase gene expression in fungi results in no signs of successful infection or lesions. Thus, α-Aminoadipate Reductase or Homocitrate Synthase can be used as a target for the identification of antibiotics, preferably antifungals. Accordingly, the present invention provides methods for the identification of compounds that inhibit α-Aminoadipate Reductase or Homocitrate 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
ALPHA-AMINOADIPATE REDUCTASE AND HOMOCITRATE SYNTHASE AS
ANTIBIOTICS

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RELATED APPLICATIONS

This application claims the benefit of U.S. Patent Application No. 10/011,146,
filed Nov. 09, 2001, and U.S. Patent Application No. 10/011,200, filed Nov. 09, 2001,
10   the contents of which are incorporated by reference.

FIELD OF THE INVENTION

The invention relates generally to methods for the identification of antibiotics,
15   preferably antifungals that affect the biosynthesis of lysine.

BACKGROUND OF THE INVENTION

Filamentous fungi are the causal agents responsible for many serious
20   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
25   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,

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Bjerkandera, Botryosphaeria, Botrytis, Ceratobasidium, Ceratocystis, Cercospora, Cercosporidium, Cerotelium, Cerrena, Chondrostereum, Chryphonectria, Chrysomyxa, Cladosporium, Claviceps, Cochliobolus, Coleosporium, Colletotrichium, Colletotrichum, Corticium, Corynespora, Cronartium,

5   *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,*

10   *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,*

15   *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,*

20   *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,*

25   *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,*

30   *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.

5   (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.

10   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. Tang et al. (Tang, C. M., J. M. Smith, et

15 al. (1994) Infect Immun 62: 5255 - 60 (PMID: 7960102)) using the related fungus, Aspergillus nidulans, observed that a mutation in para-aminobenzoic acid synthesis prevented mortality in mice, while a mutation in lysine biosynthesis had no significant effect on the mortality of the infected mice.

Therefore, it is not currently possible to determine which specific growth

20 materials may be readily obtained by a pathogen from its host, and which materials may not. Surprisingly, especially in light of the results showing that a lysine biosynthesis mutation in the filamentous fungus, Aspergillus nidulans, had no significant effect on the pathogenicity in a mouse model system (Tang, C. M., J. M. Smith, et al. (1994) Infect Immun 62: 5255 - 60 (PMID: 7960102)), we have found

25 that Magnaporthe grisea that cannot synthesize their own lysine are entirely 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 lysine biosynthesis in filamentous fungi. Thus, it has not been shown that the de novo biosynthesis of

30 lysine is essential for fungal pathogenicity. Our work with homocitrate synthase in the lysine pathway shows that the disruption of lysine biosynthesis as the result of a disruption of the gene encoding the enzyme activity, homocitrate synthase, also results in a non-pathogenic phenotype for M. grisea. Thus, it would be desirable to determine the utility of the enzymes involved in lysine 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

5   gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

SUMMARY OF THE INVENTION

10   Surprisingly, the present inventors have discovered that *in vivo* disruption of the gene encoding α-Aminoadipate Reductase and, separately, *in vivo* disruption of the gene encoding Homocitrate Synthase in *Magnaporthe grisea* each prevents or inhibits the pathogenicity of the fungus. Both of these enzymes are involved in the biosynthesis of lysine. Thus, the present inventors have discovered that α-

15   Aminoadipate Reductase and Homocitrate Synthase are each essential for normal rice blast pathogenicity, and can be used as a target for the identification of antibiotics, preferably fungicides. Accordingly, the present invention provides methods for the identification of compounds that inhibit the expression or activity of α-Aminoadipate Reductase or, separately, of Homocitrate Synthase. The methods of the invention are

20   useful for the identification of antibiotics, preferably fungicides.

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the reaction performed by α-Aminoadipate Reductase

25   (AAR1) reaction. The Substrates/Products are L-2-Aminoadipate + NADPH + ATP and the Products/Substrates are L-2-Aminoadipate 6-semialdehyde + NADP+ + AMP + pyrophosphate + H_2O. The function of the α-Aminoadipate Reductase enzyme is the interconversion of L-2-Aminoadipate, NADPH, and ATP to L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate, and H_2O. This reaction is part of the

30   lysine biosynthesis pathway.

Figure 2 shows a digital image showing the effect of AAR1 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 KO1-11. Leaf segments were imaged at five days post-inoculation.

Figure 3. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-1 and KO1-11, were grown in (A) minimal media and (B) minimal media with the addition of L-lysine, 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 KO1-11 (-- ▲--).

Figure 4 shows the reaction performed by the homocitrate synthase (HCS1) reaction. The Substrates/Products are Acetyl CoA + H_2O + 2-oxoglutarate and the Products/Substrates are 2-hydroxybutane-1,2,4-tricarboxylate + CoA. The function of the homocitrate synthase enzyme is the interconversion of Acetyl CoA, 2-oxoglutarate, and H_2O to 2-hydroxybutane-1,2,4-tricarboxylate and CoA. This reaction is part of the lysine biosynthesis pathway.

Figure 5 shows a digital image showing the effect of HCS1 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 KO1-2. Leaf segments were imaged at five days post-inoculation.

Figure 6. Verification of Gene Function by Analysis of Nutritional Requirements. Wild-type and transposon insertion strains, KO1-1 and KO1-2, were grown in (A) minimal media and (B) minimal media with the addition of L-lysine, 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 KO1-2 (-- ▲--).

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

5   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

10   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

15   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.

20   As used herein, the term "α-Aminoadipate Reductase (EC 1.2.1.31), α-Aminoadipate Reductase polypeptide, α-Aminoadipate semialdehyde dehydrogenase, or Alpha-Aminoadipate Reductase" is synonymous with "the AAR1 gene product" or the "the ASD1 gene product" and refers to an enzyme that catalyses the interconversion of L-2-Aminoadipate, NADPH, and ATP with L-2-Aminoadipate 6-

25   semialdehyde, NADP+, AMP, pyrophosphate, and H₂O.

        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.

        The term "binding" refers to a non-covalent or a covalent interaction,

30   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

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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

5   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

10   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

15   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

20   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.)

25   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

30   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.

5   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

10   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,

15   temperatures. Similar responses also result from the appropriate growth changes for other types of growth conditional phenotypes.

         As used herein, the term "H₂O" means water.

         As used herein, the term "AAR1" means a gene encoding α-Aminoadipate Reductase activity, referring to an enzyme that catalyses the interconversion of L-2-

20   Aminoadipate, NADPH, and ATP with L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate, and H₂O.

         As used herein, the term "heterologous AAR1 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

25   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* α-Aminoadipate Reductase , preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in ascending order.

         As used herein, the term "HCS1" means a gene encoding homocitrate synthase

30   activity, referring to an enzyme that catalyses the interconversion of acetyl-CoA, H₂O, and 2-oxoglutarate with 2-hydroxybutane-1,2,4-tricarboxylate and CoA.

         As used herein, the term "heterologous HCS1 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* homocitrate synthase, preferably 25%, 50%, 75%, 90%, 95%, 99% and each integer unit of activity from 10-100% in

5   ascending order.

As used herein, the term "homocitrate synthase (EC 4.1.3.21) or homocitrate synthase polypeptide" is synonymous with "the HCS1 gene product" and refers to an enzyme that catalyses the interconversion of acetyl-CoA, H_2O, and 2-oxoglutarate with 2-hydroxybutane-1,2,4-tricarboxylate and CoA.

10   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

15   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

20   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.

25   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 of α-Aminoadipate Reductase or Homocitrate Synthase, or substantially reduces the level of enzymatic activity, wherein

30   "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 "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 determining 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.

5   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.

10   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

15   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[TM]. 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.

20   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

25   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.

30   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

5   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

10   hypersensitivity or hyposensitivity.

       The term "specific binding" refers to an interaction between α-Aminoadipate Reductase and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence and/or the conformation of α-Aminoadipate Reductase.

15   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

20   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

25   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

30   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.

5   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
10   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).

15   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
20   reduction, pigment production, changes in medium acidity, and the like.

The present inventors have discovered that disruption of the AAR1 or HCS1 gene and/or gene product inhibits the pathogenicity of *Magnaporthe grisea*. Thus, the inventors are the first to demonstrate that α-Aminoadipate Reductase and Homocitrate
25   Synthase are targets for antibiotics, preferably antifungals.

Accordingly, the invention provides methods for identifying compounds that inhibit AAR1 or HCS1 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 AAR1 or HSC1 gene expression. Any compound that is a
30   ligand for α-Aminoadipate Reductase or for Homocitrate 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 at least one polypeptide selected from the group consisting of:

         i)   α-Aminoadipate Reductase; and

         ii)   Homocitrate Synthase

         with said test compound; and

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

The α-Aminoadipate Reductase or Homocitrate Synthase protein may have the amino
acid sequence of a naturally occurring α-Aminoadipate Reductase or Homocitrate

10   Synthase found in a fungus, animal, plant, or microorganism, or may have an amino
acid sequence derived from a naturally occurring sequence. Preferably the α-
Aminoadipate Reductase or Homocitrate Synthase is a fungal α-Aminoadipate
Reductase or Homocitrate Synthase. The cDNA (SEQ ID NO: 1) encoding the α-
Aminoadipate Reductase protein, the genomic DNA (SEQ ID NO: 2) encoding the

15   protein, and the polypeptide (SEQ ID NO: 3) can be found herein. The cDNA (SEQ
ID NO: 4) encoding the Homocitrate Synthase protein, the genomic DNA (SEQ ID
NO: 5) encoding the protein, and the polypeptide (SEQ ID NO: 6) can be found
herein, as well.

By "fungal α-Aminoadipate Reductase " is meant an enzyme that can be

20   found in at least one fungus, and which catalyzes the interconversion of L-2-
Aminoadipate and NADPH and ATP with L-2-Aminoadipate 6-semialdehyde,
NADP+, AMP, pyrophosphate, and H_2O. The α-Aminoadipate Reductase may be
from any of the fungi, including ascomycota, zygomycota, basidiomycota,
chytridiomycota, and lichens.

25   By "fungal Homocitrate Synthase" is meant an enzyme that can be found in at
least one fungus, and which catalyzes the interconversion of acetyl-CoA and H_2O and
2-oxoglutarate with 2-hydroxybutane-1,2,4-tricarboxylate and CoA. The homocitrate
synthase may be from any of the fungi, including ascomycota, zygomycota,
basidiomycota, chytridiomycota, and lichens.

30   In one embodiment, the α-Aminoadipate Reductase or Homocitrate Synthase
is a *Magnaporthe* α-Aminoadipate Reductase or Homocitrate 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* α-
Aminoadipate Reductase or Homocitrate Synthase is from *Magnaporthe grisea.*

5   In various embodiments, the α-Aminoadipate Reductase or Homocitrate
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

10   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

15   (*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*),

20   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 α-Aminoadipate Reductase or Homocitrate Synthase
polypeptide may be used in the methods of the invention, preferably if the fragments

25   include an intact or nearly intact epitope that occurs on the biologically active
wildtype α-Aminoadipate Reductase or Homocitrate Synthase. The fragments
comprise at least 10 consecutive amino acids of an α-Aminoadipate Reductase or
Homocitrate 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,

30   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, 770, 780, 790, 800, 810, 820, 830, 840, 850, 860, 870, 880, 890,

900, 910, 920, 930, 940, 950, 960, 970, 980, 990, 1000, 1010, 1020, 1030, 1040,

1050, 1060, 1070, 1080, 1090, 1100, 1110, 1120, 1130, 1140, 1150, 1160, 1170, or at

least 1180 consecutive amino acids residues of an α-Aminoadipate Reductase or

Homocitrate Synthase. In one embodiment, the fragment is from a *Magnaporthe* α-

5   Aminoadipate Reductase or Homocitrate Synthase. Preferably, the fragment contains

an amino acid sequence conserved among fungal α-Aminoadipate Reductases or

Homocitrate Synthases.

Polypeptides having at least 50% sequence identity with a fungal α-

Aminoadipate Reductase or Homocitrate Synthase are also useful in the methods of

10   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

15   of a fungal α-Aminoadipate Reductase or Homocitrate 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 α-Aminoadipate Reductase or Homocitrate 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* α-Aminoadipate Reductase or

20   Homocitrate 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 said test compound with at least one polypeptide selected from the

group consisting of: a polypeptide having at least ten consecutive amino acids

25   of a fungal α-Aminoadipate Reductase, a polypeptide having at least 50%

sequence identity with a fungal α-Aminoadipate Reductase, and a polypeptide

having at least 10% of the activity thereof; and

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

and said polypeptide;

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

In still another embodiment, the invention provides a method for identifying a

test compound as a candidate for a fungicide, comprising:

a) contacting said 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 homocitrate synthase, a polypeptide having at least 50% sequence identity with a fungal homocitrate synthase, and a polypeptide having at least

5   10% of the activity thereof; 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

10   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

15   candidate ligands is provided. The immobilized ligands are contacted with an α-Aminoadipate Reductase or Homocitrate Synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound α-Aminoadipate Reductase is detected. In a preferred embodiment, bound α-Aminoadipate Reductase or Homocitrate Synthase is detected using a labeled binding partner, such as a labeled

20   antibody. In a variation of this assay, α-Aminoadipate Reductase or Homocitrate 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.

25   Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit α-Aminoadipate Reductase or Homocitrate 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

30   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 α-Aminoadipate Reductase 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. α-Aminoadipate Reductase catalyzes the irreversible or reversible reaction L-2-Aminoadipate and NADPH and ATP = L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate, and H_2O (see Figure 1). Methods for detection of L-2-Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP, ATP, H_2O 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 either:

a) contacting L-2-Aminoadipate and NADPH and ATP with an α-Aminoadipate Reductase ;

b) contacting L-2-Aminoadipate and NADPH and ATP with α-Aminoadipate Reductase and said test compound; and

c) determining the change in concentration for at least one of the following: L-2-
Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,
ATP, H₂O 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.

or,

5   a) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,
and H₂O with an α-Aminoadipate Reductase ;

b) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,
and H₂O with an α-Aminoadipate Reductase and said test compound; and

c) determining the change in concentration for at least one of the following: L-2-

10   Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,
ATP, H₂O 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.

The ability of a compound to inhibit homocitrate 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. Homocitrate synthase

15   catalyzes the irreversible or reversible reaction acetyl-CoA and H₂O and 2-
oxoglutarate = 2-hydroxybutane-1,2,4-tricarboxylate and CoA (see Figure 4).
Methods for detection of 2-hydroxybutane-1,2,4-tricarboxylate, CoA, acetyl-CoA,
and/or 2-oxoglutarate, include spectrophotometry, mass spectroscopy, thin layer
chromatography (TLC) and reverse phase HPLC.

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

a) contacting acetyl-CoA and H₂O and 2-oxoglutarate with a homocitrate synthase;

b) contacting acetyl-CoA and H₂O and 2-oxoglutarate with homocitrate synthase
and said test compound; and

25   c) determining the change in concentration for at least one of the following: 2-
hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or
H₂O.

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

or,

    a) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a homocitrate
       synthase;

    b) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a homocitrate

5   synthase and said test compound; and

    c) determining the change in concentration for at least one of the following: 2-
       hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or
       H_2O.

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 α-Aminoadipate Reductase or

10 Homocitrate Synthase are also useful in the methods of the invention. For example, a
polypeptide comprising at least 100 consecutive amino acid residues of a fungal α-
Aminoadipate Reductase or Homocitrate Synthase may be used in the methods of the
invention. In addition, a polypeptide having at least 50%, 60%, 70%, 80%, 90%, 95%
or at least 98% sequence identity with a fungal α-Aminoadipate Reductase or

15 Homocitrate Synthase may be used in the methods of the invention. Most preferably,
the polypeptide has at least 50% sequence identity with a fungal α-Aminoadipate
Reductase or Homocitrate 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

20 candidate for a fungicide, comprising:

    a) contacting L-2-Aminoadipate and NADPH and ATP with a polypeptide selected
       from the group consisting of: a polypeptide having at least 50% sequence
       identity with an α-Aminoadipate Reductase, a polypeptide having at least 50%
       sequence identity with an α-Aminoadipate Reductase and having at least 10%

25   of the activity thereof, and a polypeptide comprising at least 100 consecutive
       amino acids of an α-Aminoadipate Reductase

    b) contacting L-2-Aminoadipate and NADPH and ATP with said polypeptide and
       said test compound; and

    c) determining the change in concentration for at least one of the following: L-2-

30   Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,
       ATP, H_2O 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.

or,

a) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,
and H_2O with a polypeptide selected from the group consisting of: a polypeptide
having at least 50% sequence identity with an α-Aminoadipate Reductase , a
polypeptide having at least 50% sequence identity with an α-Aminoadipate

5   Reductase and at least 10% of the activity thereof, and a polypeptide
comprising at least 100 consecutive amino acids of an α-Aminoadipate
Reductase

b) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,
and H_2O, with said polypeptide and said test compound; and

10   c) determining the change in concentration for at least one of the following, L-2-
Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,
ATP, H_2O 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.

15   Additionally, the invention provides a method for identifying a test
compound as a candidate for a fungicide, comprising:

a) contacting acetyl-CoA and H_2O and 2-oxoglutarate with a polypeptide selected
from the group consisting of: a polypeptide having at least 50% sequence
identity with a homocitrate synthase, a polypeptide having at least 50%

20   sequence identity with a homocitrate synthase and having at least 10% of the
activity thereof, and a polypeptide comprising at least 100 consecutive amino
acids of a homocitrate synthase

b) contacting acetyl-CoA and H_2O and 2-oxoglutarate with said polypeptide and
said test compound; and

25   c) determining the change in concentration for at least one of the following: 2-
hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or
H_2O.

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

or,

a) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a polypeptide

    selected from the group consisting of: a polypeptide having at least 50%

    sequence identity with a homocitrate synthase, a polypeptide having at least

    50% sequence identity with a homocitrate synthase and at least 10% of the

    activity thereof, and a polypeptide comprising at least 100 consecutive amino

    acids of a homocitrate synthase

b) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA, with said

    polypeptide and said test compound; and

c) determining the change in concentration for at least one of the following, 2-

    hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or

    H_2O.

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, α-Aminoadipate Reductase or Homocitrate 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 α-Aminoadipate Reductase or Homocitrate Synthase may be described in Jaklitsch and Kubicek (Jaklitsch, W. M. and C. P. Kubicek (1990) Biochem J *269*: 247 - 53 (PMID: 2115771)). Other methods for the purification of α-Aminoadipate Reductase or Homocitrate 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 a antibiotic, comprising:

a) measuring the expression of an α-Aminoadipate Reductase or Homocitrate

    Synthase in a cell, cells, tissue, or an organism in the absence of said

    compound;

b) contacting said cell, cells, tissue, or organism with said test compound and

    measuring the expression of said α-Aminoadipate Reductase or Homocitrate

    Synthase in said cell, cells, tissue, or organism;

c) comparing the expression of α-Aminoadipate Reductase or Homocitrate

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 α-Aminoadipate Reductase or Homocitrate Synthase can be

5   measured by detecting the AAR1 or HCS1 primary transcript or mRNA, α-

Aminoadipate Reductase or Homocitrate Synthase polypeptide, or α-Aminoadipate

Reductase or Homocitrate 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

10   Publishing and Wiley-Interscience, New York, 1995. The method of detection is not

critical to the invention. Methods for detecting AAR1 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 AAR1 or HCS1 promoter fused to a

15   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 AAR1 or HCS1 protein expression. For detection using

20   gene reporter systems, a polynucleotide encoding a reporter protein is fused in frame

with AAR1 or HCS1, 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 AAR1 or HCS1 expression or activity can then

25   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

30   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

5   (*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

10   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

15   (*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

20   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

25   whether a test compound is active against the gene identified (SEQ ID NO: 1 and SEQ ID NO: 2 for AAR1, or SEQ ID NO: 4 and SEQ ID NO: 5 for HCS1), its gene product (SEQ ID NO: 3 for AAR1 or SEQ ID NO: 6 for HCS1), or the biochemical pathway or pathways on which it functions.

In one particular embodiment, the method is performed by providing an

30   organism having a first form of the gene corresponding to either SEQ ID NO: 1 or SEQ ID NO: 2 for AAR1, or SEQ ID NO: 4 and SEQ ID NO: 5 for HCS1, either a normal form, a mutant form, a homologue, or a heterologous AAR1 or HCS1 gene that performs a similar function as AAR1 or HCS1, respectively. The first form of AAR1 or HCS1 may or may not confer a growth conditional phenotype, *i.e.*, a lysine

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 AAR1 or HCS1, 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 AAR1 or HCS1 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 α-Aminoadipate Reductase gene, and providing comparison cells having a different form of an α-Aminoadipate Reductase gene,

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

In another 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 Homocitrate Synthase gene, and providing comparison cells having a different form of a Homocitrate Synthase gene,

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 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 AAR1 gene or an HCS1 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

5   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

10   al. (1993) <u>Principles of Biochemistry</u>. 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 AAR1 or HCS1 functions, comprising:

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

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

   c) determining the growth of said cells and 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 compound indicates that said compound is a candidate for an antibiotic.

The use of multi-well plates for screening is a format that readily

15   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

20   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.

25   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

5   which AAR1 or HCS1 functions, comprising:

(a) providing paired growth media; comprising a first medium and a second

medium, wherein said second medium contains a higher level of lysine than

said first medium;

(b) contacting an organism with said test compound;

10   (c) inoculating said first and 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 second media 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

15   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*.

20

EXPERIMENTAL

Example 1

Construction of Plasmids with a Transposon Containing a Selectable Marker.

25

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

30   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

5   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) <u>Molecular Cloning, a Laboratory Manual</u>, Cold Spring

Harbor Laboratory Press.) containing 50ug/ml of hygromycin B (Sigma Chem. Co.,

10   St. Louis, MO).

Example 2
Construction of a Cosmid Library Containing Fungal Genes and a Selectable Marker

15   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) <u>Molecular Cloning, a Laboratory Manual</u>, Cold Spring Harbor

Laboratory Press. Cosmid libraries were quality checked by pulsed-field gel

electrophoresis, restriction digestion analysis, and PCR identification of single genes.

20

Example 3
Construction of Cosmids with Transposon Inserted into Fungal Genes.

          Sif Transposition into a Cosmid: Transposition of Sif into the cosmid

25   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 μg of target cosmid DNA were mixed and taken to a final volume of 20ul with

water. 1ul of transposase (TnsABC) was added to the reaction and incubated for 10

minutes at 37°C to allow the assembly reaction to happen. After the assembly

30   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.).

5

<center>Example 4</center>

<center>High Throughput Preparation and Verification of Insertion of</center>

<center>Transposon into Fungal Genes</center>

10   *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)

15 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.).

20   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* AAR1 gene was chosen for further analysis. This construct was designated cpgmra0015019g12 and it contains the SIF transposon

25 approximately between amino acids 678 and 679 relative to the *Neurospora crassa* homologue (total length - 1174 amino acids, GENBANK: 9367248 of accession number AL389890).

      As for AAR1 above, DNA sequences adjacent to the site of the insertion were collected and used to search DNA and protein databases using the BLAST algorithms

30 (Altschul *et al.* (1997) Nucleic Acids Res *25*: 3389 – 3402 (PMID: 9254694)). A single insertion of SIF into the *Magnaporthe grisea* HCS1 gene was chosen for further analysis. This construct was designated cpgmra0023008h04 and it contains the SIF transposon between amino acids 334 and 335 relative to the *Penicillium*

chrysogenum homologue (total length - 474 amino acids, GENBANK: PCAJ3630
accession number AJ223630).

Example 5

Preparation of Cosmid DNA and Transformation of the Fungus *Magnaporthe grisea*

5

Cosmid DNA from the AAR1 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

10   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 2×10^8 protoplasts/ml. 50ul

15   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

20   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 for AAR1 and are hereby
referred to as KO1-1 and KO1-11, respectively. For HCS1, two independent strains

25   were also identified and are hereby referred to as HCS1 KO1-1 and KO1-2,
respectively.

Example 6

Effect of AAR1 Transposon Insertion on *Magnaporthe* pathogenicity

30

The target fungal strains, AAR1 KO1-1 and KO1-11, 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

5   (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 AAR1 gene

10   disruption on *Magnaporthe* infection at five days post-inoculation.

Example 7

Verification of AAR1 Gene Function by Analysis of Nutritional Requirements

15   The fungal strains, KO1-1 and KO1-11, containing the AAR1 disrupted gene obtained in Example 5 were analyzed for their nutritional requirement for lysine using the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate tests for the auxotrophic requirement for 94 different metabolites. The inoculating fluid consists of 0.05% Phytagel, 0.03% Pluronic F68, 1% glucose, 23.5mM $NaNO_3$,

20   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 $MnCl_2\cdot4H_2O$, 1.8µM $FeCl_2\cdot4H_2O$, 0.71µM $CoCl_2\cdot6H_2O$, 0.64µM $CuCl_2\cdot2H_2O$, 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

25   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

30   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-lysine.

Example 8

Effect of HCS1 Transposon Insertion on *Magnaporthe* pathogenicity

The target fungal strains, HSC1 KO1-1 and KO1-2, 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 (5×10^4 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 5 shows the effects of HCS1 gene
disruption on *Magnaporthe* infection at five days post-inoculation.

Example 9

Verification of HCS1 Gene Function by Analysis of Nutritional Requirements

The fungal strains, KO1-1 and KO1-2, containing the HCS1 disrupted gene
obtained in Example 5 were analyzed for their nutritional requirement for lysine using
the PM5 phenotype microarray from Biolog, Inc. (Hayward, CA). The PM5 plate
tests for the auxotrophic requirement for 94 different metabolites. The inoculating
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
$CoCl_2 \cdot 6H2O$, 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 2×10^5 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

5   showing both the mutant fungi and the wild-type control in the absence (Figure 6A) and presence (Figure 6B) of L-lysine.

Example 10

Cloning and Expression Strategies, Extraction and Purification of α-Aminoadipate
10   Reductase or Homocitrate Synthase protein.

The following protocol may be employed to obtain a purified α-Aminoadipate Reductase protein.

Cloning and expression strategies:
15   An AAR1or HCS1 cDNA gene can be cloned into *E. coli* (pET vectors-
Novagen), Baculovirus (Pharmingen) and Yeast (Invitrogen) expression
vectors containing His/fusion protein tags. Evaluate the expression of
recombinant protein by SDS-PAGE and Western blot analysis.

20   **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
25   recombinant protein by activity assay.

Purification:
Purify recombinant protein by Ni-NTA affinity chromatography (Qiagen).
Purification protocol: perform all steps at 4°C:
30   • 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 11

Assays for Testing Binding of Test Compounds to α-Aminoadipate Reductase or Homocitrate Synthase

The following protocol may be employed to identify test compounds that bind to the α-Aminoadipate Reductase or Homocitrate Synthase protein.

- Purified full-length α-Aminoadipate Reductase or Homocitrate Synthase polypeptide with a His/fusion protein tag (Example 10) 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, Jaklitsch, W. M. and C. P. Kubicek (1990) Biochem J 269: 247 – 53 (PMID: 2115771)) for binding of radiolabeled ^{14}C-α-Aminoadipic acid (Sigma-Aldrich Co.) to the bound AAR1, or for binding of radiolabeled (acetyl-1-^{14}C)-coenzyme A (Sigma-Aldrich Co.) to the bound HCS1.

- Screening of test compounds is performed by adding test compound and ^{14}C-α-Aminoadipic acid (Sigma-Aldrich Co.) to the wells of the HisGrab™ plate containing bound AAR1, or performed by adding test compound and (acetyl-1-^{14}C)-coenzyme A (Sigma-Aldrich Co.) to the wells of the HisGrab™ plate containing bound HCS1.

- 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 α-Aminoadipate Reductase or Homocitrate Synthase is screened in the same way. A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the AAR1 or HCS1 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 AAR1 or HCS1 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 10 above.

5   Test compounds that bind AAR1 or HCS1 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

10   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.

15

Example 12

Assays for Testing Inhibitors or Candidates for Inhibition of α-Aminoadipate Reductase or Homocitrate Synthase Activity

20   The enzymatic activity of α-Aminoadipate Reductase or Homocitrate Synthase is determined in the presence and absence of candidate compounds in a suitable reaction mixture, such as described by Gray and Bhattacharjee (Gray, GS and Bhattacharjee, JK (1976) Can J Microbiol *22*: 1664 - 7 (PMID: 10066)), or Jaklitsch, W. M. and C. P. Kubicek (1990) Biochem J *269*: 247 – 53 (PMID: 2115771).

25   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* α-Aminoadipate Reductase or Homocitrate Synthase is determined in the presence and absence of candidate compounds in a suitable reaction

30   mixture, such as described by Gray and Bhattacharjee (Gray, GS and Bhattacharjee, JK (1976) Can J Microbiol *22*: 1664 - 7 (PMID: 10066)), or Jaklitsch, W. M. and C. P. Kubicek (1990) Biochem J *269*: 247 - 53 (PMID: 2115771). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the AAR1 or

HCS1 gene into a protein expression vector that adds a His-Tag when expressed (see
Example 10). Oligonucleotide primers are designed to amplify a portion of the AAR1
or HCS1 gene using polymerase chain reaction amplification method. The DNA
fragment encoding a polypeptide of 10-50 amino acids is cloned into an expression

5   vector, expressed and purified as described in Example 10 above.

Test compounds identified as inhibitors of AAR1 or HCS1 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.*

10   (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

15   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.

20   Example 13
          Assays for Testing Compounds or Candidates for Compounds That Alter the
          Expression of an α-Aminoadipate Reductase or Homocitrate Synthase Gene

          Magnaporthe grisea fungal cells are grown under standard fungal growth

25   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.

30   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

5   RNA samples as described (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory

Manual, Cold Spring Harbor Laboratory Press) using a radiolabeled fragment of the

AAR1 or HCS1 gene as a probe. Test compounds resulting in a reduced level of

AAR1 or HCS1 mRNA relative to the untreated control sample are identified as

candidate antibiotic compounds.

10

Example 14

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a

Mutant Form of α-Aminoadipate Reductase or Homocitrate Synthase with No

Activity

15

Magnaporthe grisea fungal cells containing a mutant form of the AAR1 or

HCS1 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

20   harvested from cultures grown on complete agar medium containing 4mM L-lysine

(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-lysine to a concentration of $2x10^5$ spores per ml.

25   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

30   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:

5   7749303)).

Example 15

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a

Mutant Form of α-Aminoadipate Reductase or Homocitrate Synthase with Reduced

10   Activity

Magnaporthe grisea fungal cells containing a mutant form of the AAR1 or

HCS1 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

15   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 4mM

20   L-lysine (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 2×10^5 spores per ml. Approximately 4×10^4 spores are added to each

well of 96-well plates to which a test compound is added (at varying concentrations).

25   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

30   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)).

5   Example 16

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

Magnaporthe grisea fungal cells containing a mutant form of a gene in the
10   lysine biosynthetic pathway (*e.g.* HCS1 (E.C. 4.1.3.21)) 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
4mM L-lysine (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
15   hemacytometer and spore suspensions are prepared in a minimal growth medium
containing 100 µM L-lysine 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
20   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
25   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
30   (PMID: 7749303)).

Example 17

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

Magnaporthe grisea fungal cells containing a mutant form of a gene in the

5   lysine biosynthetic pathway (*e.g.* HCS1 (E.C. 4.1.3.21)), 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

10   art (Sambrook *et al.* (1989) <u>Molecular Cloning, a Laboratory Manual</u>, 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 4mM L-lysine (Sigma-Aldrich Co.) after growth

15   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 2×10^5 spores per ml. Approximately
4×10^4 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

20   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

25   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

30   found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID:
7749303)).

Example 18

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a

Fungal AAR1 and a Second Fungal Strain Containing a Heterologous AAR1 Gene

5

Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking

a functional AAR1 gene and containing an AAR1 gene from *Penicillium*

chrysogenum (Genbank accession Y13967, 56% sequence identity) are grown under

standard fungal growth conditions that are well known and described in the art. An

10   *M. grisea* strain carrying a heterologous AAR1 gene is made as follows:

- An *M. grisea* strain is made with a nonfunctional AAR1 gene, such as one
  containing a transposon insertion in the native gene (see Examples 4 and 5).

- A construct containing a heterologous AAR1 gene is made by cloning the AAR1
  gene from *Penicillium chrysogenum* into a fungal expression vector containing a
15   *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
20   AAR1 gene (see Example 5). Transformants are selected on minimal agar
  medium lacking L-lysine. Only transformants carrying a functional AAR1 gene
  will grow.

Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous

form of AAR1 are grown under standard fungal growth conditions that are well

25   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 2×10^5 spores per ml. Approximately 4×10^4 spores or cells are

30   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.

5   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 AAR1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology *26*: 177 - 221 (PMID: 7749303)).

10

Example 19

In Vivo Cell Based Assay Screening Protocol with a Fungal Strain Containing a Fungal HCS1 and a Second Fungal Strain Containing a Heterologous HCS1 Gene

15   Wild-type *Magnaporthe grisea* fungal cells and *M. grisea* fungal cells lacking a functional HCS1 gene and containing a HCS1 gene from *Thermus aquaticus* (Genbank accession O87198, 56% sequence identity) are grown under standard, fungal growth conditions that are well known and described in the art. An *M. grisea* strain carrying a heterologous HCS1 gene is made as follows:

20   •   An *M. grisea* strain is made with a nonfunctional HCS1 gene, such as one containing a transposon insertion in the native gene (see Examples 4 and 5).

       •   A construct containing a heterologous HCS1 gene is made by cloning the HCS1 gene from *Thermus aquaticus* into a fungal expression vector containing a *trpC* promoter and terminator (*e.g.* pCB1003, Carroll *et al.* (1994) Fungal Gen News

25   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 HCS1 gene (see Example 5). Transformants are selected on minimal agar

30   medium lacking L-lysine. Only transformants carrying a functional HCS1 gene will grow.

       Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of HCS1 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

5   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

10   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

15   heterologous strains are identified as potential antifungal compounds with specificity

to the native or heterologous HCS1 gene products. Similar protocols may be found in

Kirsch and DiDomenico ((1994) Biotechnology 26: 177 - 221 (PMID: 7749303)).

20   Example 20
                      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

25   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 4mM L-lysine (Sigma-

Aldrich Co.) to a concentration of $2x10^5$ spores per ml. The minimal growth media

30   contains carbon, nitrogen, phosphate, and sulfate sources, and magnesium, calcium,

and trace elements (for example, see inoculating 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 4mM L-lysine. Test compounds are added to wells containing spores in

minimal media and minimal media containing L-lysine. The total volume in each

well is 200μl. Both minimal media and L-lysine containing media wells with no test

compound are provided as controls. The plates are incubated at 25°C for seven days

5   and optical density measurements at 590nm are taken daily. A compound is identified

as a candidate for an antibiotic acting against the lysine biosynthetic pathway when

the observed growth in the well containing minimal media is less than the observed

growth in the well containing L-lysine as a result of the addition of the test compound.

Similar protocols may be found in Kirsch and DiDomenico ((1994) Biotechnology

10   *26*: 177 - 221 (PMID: 7749303)).

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

15   to exemplify various specific embodiments of the invention and do not limit its scope

in any manner.

CLAIMS

What is claimed is:

1. A method for identifying a test compound as a candidate for an antibiotic,

5   comprising:

       a) contacting an α-Aminoadipate Reductase polypeptide with said test compound;

          and

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

          said α-Aminoadipate Reductase polypeptide;

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

2. The method of claim 1, wherein said α-Aminoadipate Reductase polypeptide is a

       fungal α-Aminoadipate Reductase polypeptide.

15   3. The method of claim 1, wherein said α-Aminoadipate Reductase polypeptide is a

       Magnaporthe α-Aminoadipate Reductase polypeptide.

4. The method of claim 1, wherein said α-Aminoadipate Reductase polypeptide is

       SEQ ID NO: 3.

20

5. A method for determining whether a compound identified as an antibiotic candidate

       by the method of claim 1 has antifungal activity, further comprising:

       contacting a fungus or fungal cells with said antibiotic candidate and detecting the

       decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

25

6. A method for identifying a test compound as a candidate for an antibiotic,

comprising:

   a) contacting said test compound with at least one polypeptide selected from the

   group consisting of: a polypeptide having at least ten consecutive amino acids

5   of a fungal α-Aminoadipate Reductase, a polypeptide having at least 50%

   sequence identity with a fungal α-Aminoadipate Reductase, and a polypeptide

   having at least 10% of the activity thereof; and

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

   and said polypeptide;

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

7. A method for determining whether a compound identified as an antibiotic candidate

   by the method of claim 6 has antifungal activity, further comprising:

   contacting a fungus or fungal cells with said antibiotic candidate and detecting a

15   decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

8. A method for identifying a test compound as a candidate for an antibiotic,

   comprising:

   a) contacting L-2-Aminoadipate and NADPH and ATP with an α-Aminoadipate

20   Reductase;

   b) contacting L-2-Aminoadipate and NADPH and ATP with α-Aminoadipate

   Reductase and said test compound; and

   c) determining the change in concentration for at least one of the following: L-2-

   Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,

25   ATP, H₂O 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.

9. The method of claim 8, wherein said α-Aminoadipate Reductase is a fungal α-

5   Aminoadipate Reductase.

10. The method of claim 8, wherein said α-Aminoadipate Reductase is a *Magnaporthe* α-Aminoadipate Reductase.

10   11. The method of claim 8, wherein said α-Aminoadipate Reductase is SEQ ID NO:

3.

12. A method for determining whether a compound identified as an antibiotic candidate by the method of claim 8 has antifungal activity, further comprising:

15   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.

13. A method for identifying a test compound as a candidate for an antibiotic, comprising:

20   a) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate, and H_2O with an α-Aminoadipate Reductase;

b) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate, and H_2O with an α-Aminoadipate Reductase and said test compound; and

c) determining the change in concentration for at least one of the following: L-2-

Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,

ATP, H₂O and/or pyrophosphate;

wherein a change in concentration for any of the above substances between steps (a)

5   and (b) indicates that said test compound is a candidate for an antibiotic.

14. The method of claim 13, wherein said α-Aminoadipate Reductase is a fungal α-

Aminoadipate Reductase.

10   15. The method of claim 13, wherein said α-Aminoadipate Reductase is a

Magnaporthe α-Aminoadipate Reductase.

16. The method of claim 13, wherein said α-Aminoadipate Reductase is SEQ ID NO:

3.

15

17. A method for determining whether a compound identified as an antibiotic

candidate by the method of claim 13 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.

20

18. A method for identifying a test compound as a candidate for an antibiotic,

comprising:

a) contacting L-2-Aminoadipate and NADPH and ATP with a polypeptide selected

from the group consisting of: a polypeptide having at least 50% sequence

25   identity with an α-Aminoadipate Reductase, a polypeptide having at least 50%

sequence identity with an α-Aminoadipate Reductase and having at least 10%

of the activity thereof, and a polypeptide comprising at least 100 consecutive

amino acids of an α-Aminoadipate Reductase

b) contacting L-2-Aminoadipate and NADPH and ATP with said polypeptide and

5   said test compound; and

c) determining the change in concentration for at least one of the following: L-2-

Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,

ATP, H₂O and/or pyrophosphate;

wherein a change in concentration for any of the above substances between steps (a)

10   and (b) indicates that said test compound is a candidate for an antibiotic.

19. A method for identifying a test compound as a candidate for an antibiotic,

comprising:

a) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,

15   and H₂O with a polypeptide selected from the group consisting of: a polypeptide

having at least 50% sequence identity with an α-Aminoadipate Reductase, a

polypeptide having at least 50% sequence identity with an α-Aminoadipate

Reductase and at least 10% of the activity thereof, and a polypeptide comprising

at least 100 consecutive amino acids of an α-Aminoadipate Reductase

20   b) contacting L-2-Aminoadipate 6-semialdehyde, NADP+, AMP, pyrophosphate,

and H₂O, with said polypeptide and said test compound; and

c) determining the change in concentration for at least one of the following: L-2-

Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP+, NADPH, AMP,

ATP, H₂O 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.

20. A method for identifying a test compound as a candidate for an antibiotic,

5   comprising:

a) measuring the expression of an α-Aminoadipate Reductase in a cell, cells,

tissue, or an organism in the absence of said compound;

b) contacting said cell, cells, tissue, or organism with said test compound and

measuring the expression of said α-Aminoadipate Reductase in said fungus or

10   fungal cell;

c) comparing the expression of α-Aminoadipate Reductase 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.

15   21. The method of claim 20 wherein said a cell, cells, tissue, or organism is, or is

derived from a fungus.

22. The method of claim 20 wherein said cell, cells, tissue, or organism is, or is

derived from a *Magnaporthe* fungus or fungal cell.

20

23. The method of claim 20, wherein said α-Aminoadipate Reductase is SEQ ID NO:

3.

24. The method of claim 20, wherein the expression of α-Aminoadipate Reductase is

25   measured by detecting AAR1 mRNA.

25. The method of claim 20, wherein the expression of α-Aminoadipate Reductase is

    measured by detecting α-Aminoadipate Reductase polypeptide.

26. A method for identifying a test compound as a candidate for an antibiotic,

    comprising:

5   a) providing cells having one form of an α-Aminoadipate Reductase gene, and

          providing comparison cells having a different form of an α-Aminoadipate

          Reductase gene,

       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

10   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.

15   27. The method of claim 26 wherein the cells are fungal cells.

     28. The method of claim 26 wherein the cells are *Magnaporthe* cells.

     29. The method of claim 26 wherein said form and said comparison form of the α-

20   Aminoadipate Reductase are fungal α-Aminoadipate Reductases.

     30. The method of claim 26, wherein at least one form is a *Magnaporthe* α-

        Aminoadipate Reductase.

31. The method of claim 26 wherein said form and said comparison form of the α-

Aminoadipate Reductase are non-fungal α-Aminoadipate Reductases.

5   32. The method of claim 26 wherein one form of the α-Aminoadipate Reductase is a

fungal α-Aminoadipate Reductase, and the other form is a non-fungal α-

Aminoadipate Reductase.

33. 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 lysine biochemical and/or genetic

pathway and providing comparison cells having a different form of said gene.

b)   contacting said cells and comparison cells with a said test compound,

c)   determining the growth of said cells and comparison cells in the presence of said

test compound;

wherein a difference in growth between said cells and said comparison cells in the

10   presence of said compound indicates that said compound is a candidate for an

antibiotic.

34. The method of claim 33 wherein the cells are fungal cells.

15   35. The method of claim 33 wherein the cells are *Magnaporthe* cells.

36. The method of claim 33 wherein said form and said comparison form of the lysine

biosynthesis gene are fungal lysine biosynthesis genes.

37. The method of claim 33, wherein at least one form is a *Magnaporthe* lysine

biosynthesis gene.

5   38. The method of claim 33 wherein said form and said comparison form of the lysine

biosynthesis genes are non-fungal lysine biosynthesis genes.

39. The method of claim 33 wherein one form of the lysine biosynthesis gene is a

fungal lysine biosynthesis gene, and the other form is a non-fungal lysine

10   biosynthesis gene.

40. A method for determining whether a test compound identified as an antibiotic

candidate by the method of claim 33 has antifungal activity, further comprising:

contacting a fungus or fungal cells with said antibiotic candidate and detecting a

15   decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

41. 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

20   medium, wherein said second medium contains a higher level of lysine than

said first medium;

(b) contacting an organism with said test compound;

(c) inoculating said first and 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 second media

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

42. The method of claim 41, wherein said organism is a fungus.

5

43. The method of claim 41, wherein said organism is *Magnaporthe.*

44. An isolated polynucleotide comprising a nucleotide sequence that encodes a

polypeptide of SEQ ID NO: 3.

10

45. The polynucleotide of claim 44 comprising the nucleotide sequence of SEQ ID

NO: 1.

46. An expression cassette comprising the polynucleotide of claim 45.

15

47. The isolated polynucleotide of claim 44 comprising a nucleotide sequence of at

least 50 to at least 95% sequence identity to SEQ ID NO: 1.

48. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO:

20   3.

49. A polypeptide comprising the amino acid sequence of SEQ ID NO: 3.

50. A method for identifying a test compound as a candidate for an antibiotic,

25   comprising:

a) contacting a homocitrate synthase polypeptide with said test compound; and

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

said homocitrate synthase polypeptide;

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

5   51. The method of claim 50, wherein said homocitrate synthase polypeptide is a

fungal homocitrate synthase polypeptide.

52. The method of claim 50, wherein said homocitrate synthase polypeptide is a

Magnaporthe homocitrate synthase polypeptide.

10

53. The method of claim 50, wherein said homocitrate synthase polypeptide is SEQ

ID NO: 6.

54. A method for determining whether a compound identified as an antibiotic

15   candidate by the method of claim 50 has antifungal activity, further comprising:

contacting a fungus or fungal cells with said antibiotic candidate and detecting the

decrease in growth, viability, or pathogenicity of said fungus or fungal cells.

55. A method for identifying a test compound as a candidate for an antibiotic,

20   comprising:

a) contacting said 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 homocitrate synthase, a polypeptide having at least 50% sequence

identity with a fungal homocitrate synthase, and a polypeptide having at least

25   10% of the activity thereof; 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.

5   56. A method for determining whether a compound identified as an antibiotic

candidate by the method of claim 55 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.

10   57. A method for identifying a test compound as a candidate for an antibiotic,

comprising:

a) contacting acetyl-CoA and H_2O and 2-oxoglutarate with a homocitrate synthase;

b) contacting acetyl-CoA and H_2O and 2-oxoglutarate with a homocitrate synthase

and said test compound; and

15   c) determining the change in concentration for at least one of the following: 2-

hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or

H_2O;

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.

20

58. The method of claim 57, wherein said homocitrate synthase is a fungal

homocitrate synthase.

59. The method of claim 57, wherein said homocitrate synthase is a *Magnaporthe*

25   homocitrate synthase.

60. The method of claim 57, wherein said homocitrate synthase is SEQ ID NO: 6.

61. A method for determining whether a compound identified as an antibiotic

5   candidate by the method of claim 57 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.

62. A method for identifying a test compound as a candidate for an antibiotic,

10   comprising:

a) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a homocitrate

synthase;

b) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a homocitrate

synthase and said test compound; and

15   c) determining the change in concentration for at least one of the following: 2-

hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or

H_2O;

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.

20

63. The method of claim 62, wherein said homocitrate synthase is a fungal

homocitrate synthase.

64. The method of claim 62, wherein said homocitrate synthase is a *Magnaporthe*

25   homocitrate synthase.

65. The method of claim 62, wherein said homocitrate synthase is SEQ ID NO: 6.

66. A method for determining whether a compound identified as an antibiotic

5   candidate by the method of claim 62 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.

67. A method for identifying a test compound as a candidate for an antibiotic,

10   comprising:

a) contacting acetyl-CoA and H_2O and 2-oxoglutarate with a polypeptide selected

from the group consisting of: a polypeptide having at least 50% sequence

identity with a homocitrate synthase, a polypeptide having at least 50%

sequence identity with a homocitrate synthase and having at least 10% of the

15   activity thereof, and a polypeptide comprising at least 100 consecutive amino

acids of a homocitrate synthase;

b) contacting acetyl-CoA and H_2O and 2-oxoglutarate with said polypeptide and

said test compound; and

c) determining the change in concentration for at least one of the following: 2-

20   hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or

H_2O;

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.

68. A method for identifying a test compound as a candidate for an antibiotic, comprising:

   a) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA with a polypeptide selected from the group consisting of: a polypeptide having at least 50%

5   sequence identity with a homocitrate synthase, a polypeptide having at least 50% sequence identity with a homocitrate synthase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a homocitrate synthase;

   b) contacting 2-hydroxybutane-1,2,4-tricarboxylate and CoA, with said

10   polypeptide and said test compound; and

   c) determining the change in concentration for at least one of the following: 2-hydroxybutane-1,2,4-tricarboxylate, 2-oxoglutarate, acetyl-CoA, CoA, and/or H_2O;

   wherein a change in concentration for any of the above substances between steps (a)

15   and (b) indicates that said test compound is a candidate for an antibiotic.

69. A method for identifying a test compound as a candidate for an antibiotic, comprising:

   a) measuring the expression of a homocitrate synthase in a cell, cells, tissue, or an

20   organism in the absence of said compound;

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

   c) comparing the expression of homocitrate 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.

70. The method of claim 69 wherein said a cell, cells, tissue, or organism is, or is

5   derived from a fungus.

71. The method of claim 69 wherein said cell, cells, tissue, or organism is, or is

derived from a *Magnaporthe* fungus or fungal cell.

10   72. The method of claim 69, wherein said homocitrate synthase is SEQ ID NO: 3.

73. The method of claim 69, wherein the expression of homocitrate synthase is

measured by detecting HCS1 mRNA.

15   74. The method of claim 69, wherein the expression of homocitrate synthase is

measured by detecting homocitrate synthase polypeptide.

75. A method for identifying a test compound as a candidate for an antibiotic,

comprising:

a) providing cells having one form of a homocitrate synthase gene, and providing

comparison cells having a different form of a homocitrate synthase gene,

20   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.

76. The method of claim 75 wherein the cells are fungal cells.

77. The method of claim 75 wherein the cells are *Magnaporthe* cells.

78. The method of claim 75 wherein said form and said comparison form of the

homocitrate synthase are fungal homocitrate synthases.

79. The method of claim 75, wherein at least one form is a *Magnaporthe* homocitrate

synthase.

80. The method of claim 75 wherein said form and said comparison form of the

homocitrate synthase are non-fungal homocitrate synthases.

81. The method of claim 75 wherein one form of the homocitrate synthase is a fungal

homocitrate synthase, and the other form is a non-fungal homocitrate synthase.

82. 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 lysine

biochemical and/or genetic pathway and providing comparison

cells having a different form of said gene.

b)   contacting said cells and comparison cells with a said test

compound,

c)   determining the growth of said cells and 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 compound indicates that said compound is a candidate for

an antibiotic.

83. The method of claim 82 wherein the cells are fungal cells.

84. The method of claim 82 wherein the cells are *Magnaporthe* cells.

5

85. The method of claim 82 wherein said form and said comparison form of the lysine

biosynthesis gene are fungal lysine biosynthesis genes.

86. The method of claim 82, wherein at least one form is a *Magnaporthe* lysine

10   biosynthesis gene.

87. The method of claim 82 wherein said form and said comparison form of the lysine

biosynthesis genes are non-fungal lysine biosynthesis genes.

15   88. The method of claim 82 wherein one form of the lysine biosynthesis gene is a

fungal lysine biosynthesis gene, and the other form is a non-fungal lysine

biosynthesis gene.

89. A method for determining whether a test compound identified as an antibiotic

candidate by the method of claim 82 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.

5

90. 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 lysine than

10   said first medium;

(b) contacting an organism with said test compound;

(c) inoculating said first and 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 second media

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

91. The method of claim 90, wherein said organism is a fungus.

92. The method of claim 90, wherein said organism is *Magnaporthe*.

20

93. An isolated polynucleotide comprising a nucleotide sequence that encodes a

polypeptide of SEQ ID NO: 6.

94. The polynucleotide of claim 93 comprising the nucleotide sequence of SEQ ID

25   NO: 4.

95. An expression cassette comprising the polynucleotide of claim 94.

96. The isolated polynucleotide of claim 93 comprising a nucleotide sequence of at

5   least 50 to at least 95% sequence identity to SEQ ID NO: 4.

97. A polypeptide consisting essentially of the amino acid sequence of SEQ ID NO: 6.

98.   A polypeptide comprising the amino acid sequence of SEQ ID NO: 6.

10

Figure 1

L-2-Aminoadipate + NADPH + ATP

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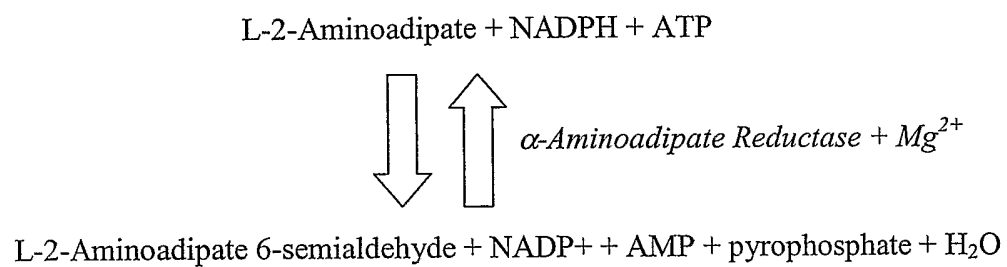
Figure 2

Negative
Control

Guy11

KO1-1

KO1-11

Figure 3

A.

B.

Figure 4

Acetyl CoA + H$_2$O + 2-oxoglutarate

⇓ ⇑ *Homocitrate synthase*

2-hydroxybutane-1,2,4-tricarboxylate + CoA
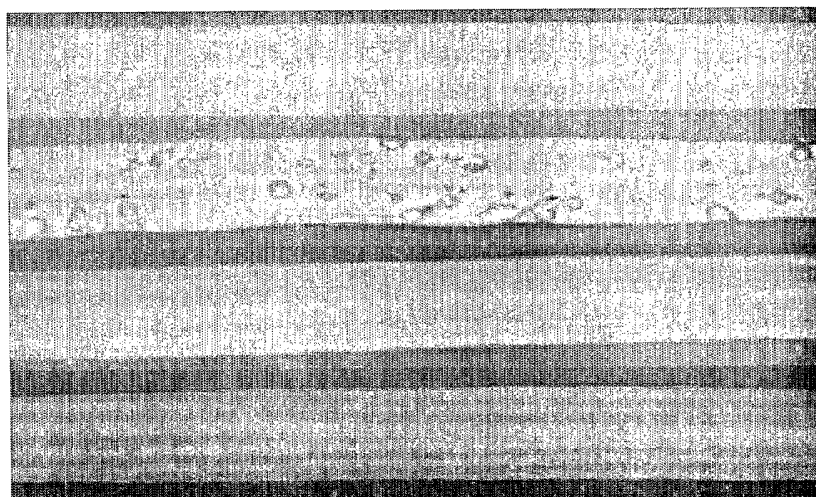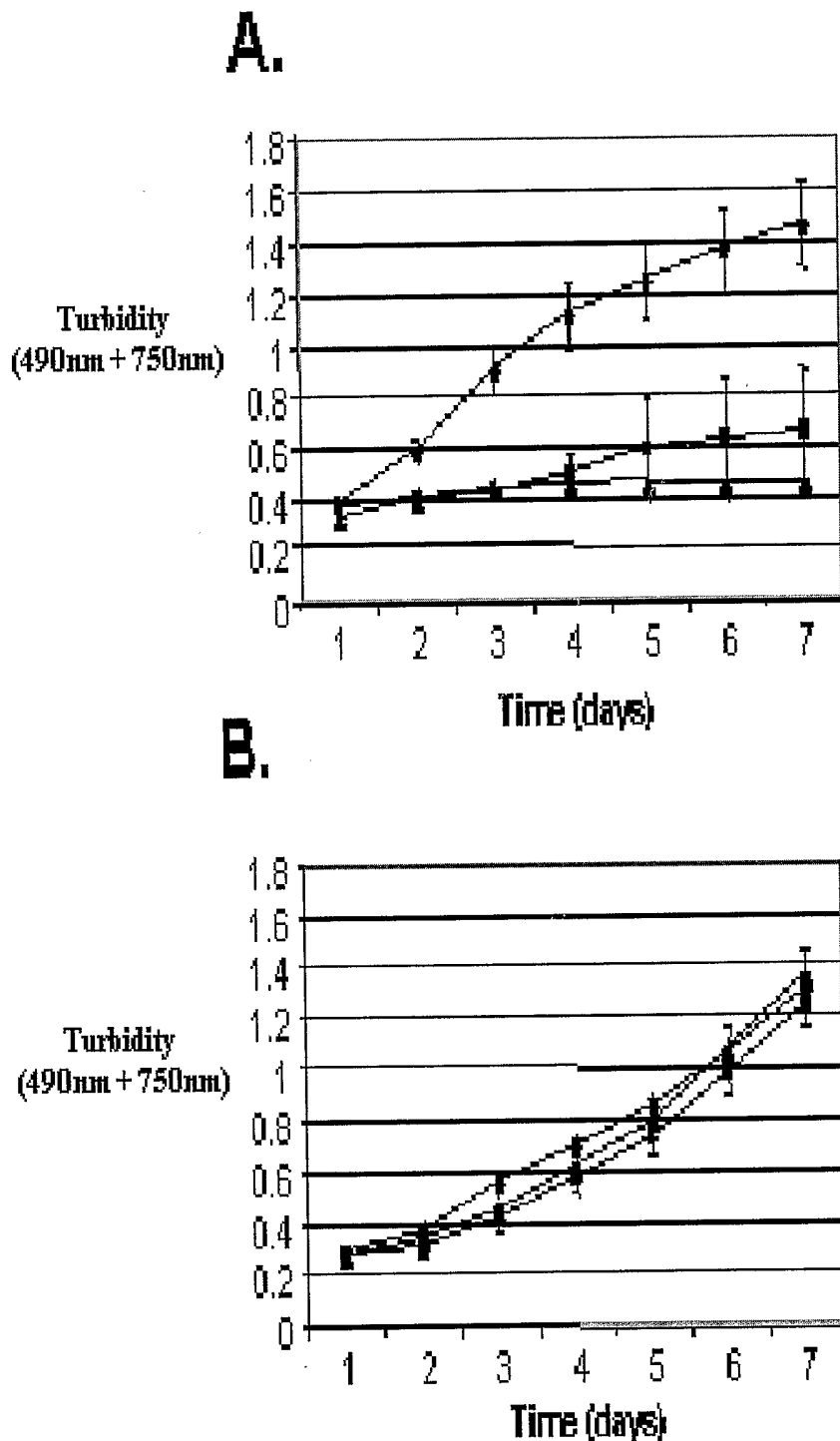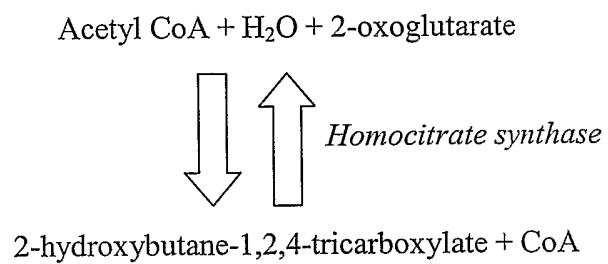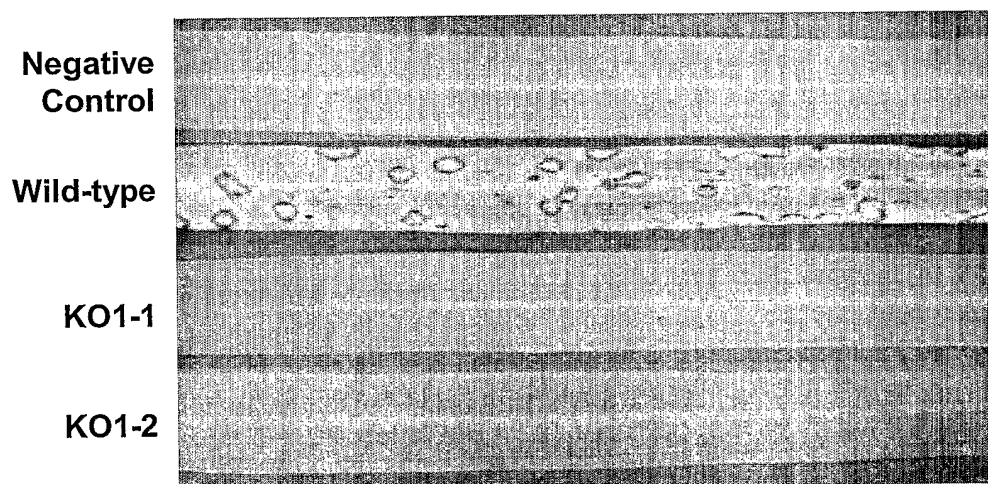
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Figure 5

Figure 6

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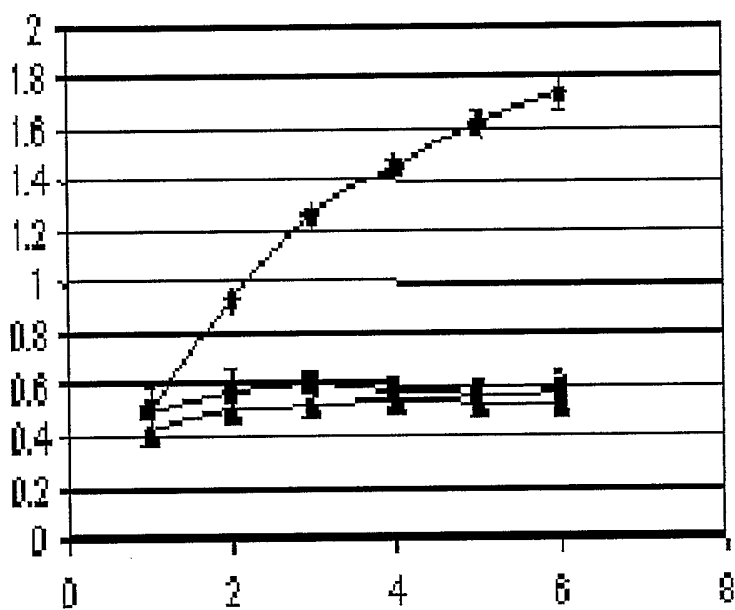
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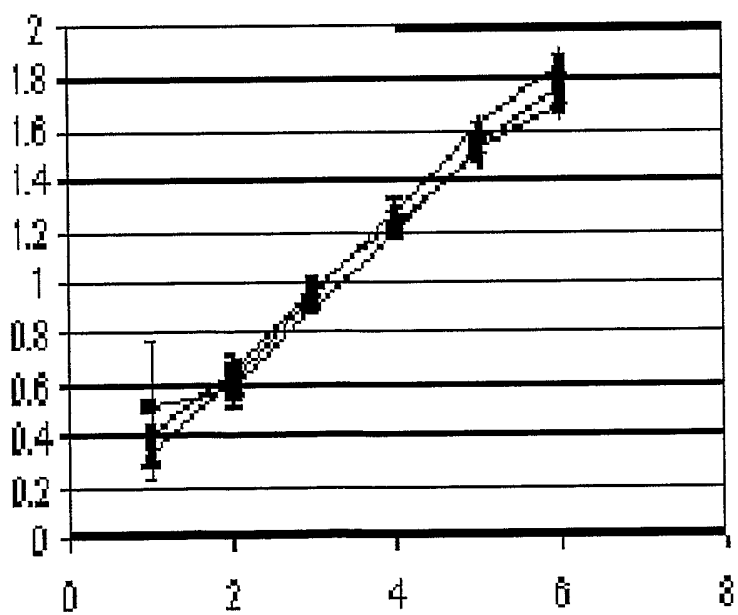
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6

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7

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                20   25   30

Pro Glu Arg Val Cys Val Ile Glu Thr Glu Ser Ser Glu Ala Pro Glu
            35   40   45

Arg Ile Phe Thr Tyr Lys Gln Ile Phe Glu Ala Ser Asn Val Leu Ala
        50   55   60

His Tyr Leu His Asp Ala Gly Val Thr Asn Gly Asp Val Val Met Ile
65   70   75   80

Trp Ala His Arg Ser Val Asp Leu Val Val Ser Ile Met Gly Val Leu
                85   90   95

Ala Ala Gly Ala Thr Phe Ser Val Leu Asp Pro Leu Tyr Pro Pro Ser
                100   105   110

Arg Gln Gln Ile Tyr Leu Glu Val Ser Gly Pro Thr Ala Leu Val Gln
            115   120   125

Ile Ala Arg Ala Thr Asp Glu Ala Gly Pro Leu Ala Pro Leu Val Arg
        130   135   140

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

Arg Ile Gly Asp Asp Gly His Leu Ser Gly Gly Glu Ile Asn Gly Ala
                165   170   175

Asp Val Phe Ala Ser Val Arg Gly Lys Ala Ser Ser Pro Pro Ala Asp
                180   185   190

Ile Glu Val Gly Pro Asp Ser Asn Pro Thr Leu Ser Phe Thr Ser Gly
            195   200   205

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Ser Glu Gly Arg Pro Lys Gly Val Leu Gly Arg His Tyr Ser Leu Ala
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Lys Tyr Phe Arg Trp Met Ala Glu Thr Phe Gly Met Gly Glu Glu Ser
225              230              235              240

Arg Phe Thr Leu Leu Ser Gly Ile Ala His Asp Pro Val Gln Arg Asp
            245              250              255

Ile Phe Thr Pro Leu Tyr Leu Gly Ala Arg Leu Leu Val Pro Ser Lys
            260              265              270

Glu Asn Ile Ala His Glu Arg Leu Ala Glu Trp Phe Lys Arg Phe Glu
        275              280              285

Pro Thr Val Thr His Leu Thr Pro Ala Met Gly Gln Ile Leu Val Gly
    290              295              300

Gly Ala Thr Ala Gln Phe Pro Ala Leu Lys Thr Ala Tyr Phe Val Gly
305              310              315              320

Asp Val Leu Thr Thr Arg Asp Cys Arg Ser Leu Arg Glu Leu Ala Ala
            325              330              335

Asn Val Asp Ile Val Asn Met Tyr Gly Thr Thr Glu Thr Ser Arg Ala
            340              345              350

Val Ser Tyr Tyr Lys Ile Pro Asn Arg Ala Ser Asp Pro Asp Phe Leu
        355              360              365

Glu Arg Leu Gly Lys Asp Thr Ile Pro Ala Gly Thr Gly Met Glu Asn
    370              375              380

Val Gln Leu Leu Val Val Asn Arg Glu Asp Arg Thr Lys Leu Cys Gly
385              390              395              400

Ile Gly Glu Val Gly Glu Ile Tyr Val Arg Ala Ala Gly Leu Ala Glu
            405              410              415

Gly Tyr Lys Gly Asp Pro Ala Leu Asn Glu Gln Lys Phe Leu Met Asn
        420              425              430
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10

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Trp Phe Val Asp Asn Asn Lys Trp Val Glu Ala Asp Arg Val His Pro
        435                 440                 445


Thr Lys Asp Ala Ala Trp Arg Lys Tyr Tyr Lys Gly Pro Leu Asp Arg
        450                 455                 460


Leu Tyr Arg Thr Gly Asp Leu Gly Lys Tyr Leu Asp Ser Gly Asp Val
465                 470                 475                 480


Glu Cys Thr Gly Arg Ala Asp Asp Gln Val Lys Ile Arg Gly Phe Arg
                485                 490                 495


Ile Glu Leu Asn Asp Ile Asp Ser Asn Leu Ser Gln Ser Ser Leu Ile
        500                 505                 510


Arg Asp Cys Lys Thr Leu Val Arg Arg Asp Lys Asn Glu Glu Pro Lys
        515                 520                 525


Leu Val Ser Tyr Val Val Pro Glu Leu Lys Gln Trp Pro Gln Trp Leu
        530                 535                 540


Lys Leu His Gly Tyr Glu Asp Ala Glu Asp Asp Glu Gly Thr Gln Ile
545                 550                 555                 560


Gly Ala Thr Lys Val Tyr Phe Lys Arg Tyr Arg Arg Met Gln Ala Glu
                565                 570                 575


Leu Arg Asp His Leu Lys Ser Arg Leu Pro Thr Tyr Ala Val Pro Ser
                580                 585                 590


Ile Phe Ile Val Leu Glu Lys Leu Pro Leu Asn Pro Asn Gly Lys Val
        595                 600                 605


Asp Lys Pro Asn Leu Pro Phe Pro Asp Ile Ala Glu Gln Thr Ala Glu
        610                 615                 620


Ala Ser Ser Glu Glu Ile Glu Arg Trp Glu Ser Leu Thr Glu Thr Glu
625                 630                 635                 640


Arg Ala Val Ala Thr Arg Trp Ala Ala Leu Ile Gln Gly Leu Asn Glu
                645                 650                 655


Lys Ser Ile Ala Pro Asp Asn His Phe Phe Asp Leu Gly Gly His Ser
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11

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                660                        665                        670


Ile  Leu  Ala  Gln  Gln  Met  Leu  Leu  Asp  Val  Arg  Lys  Gln  Met  Gly  Ala
          675                      680                      685


Asn  Val  Ser  Ile  Asn  Thr  Leu  Tyr  Glu  Asn  Pro  Thr  Leu  Gly  Ala  Phe
     690                      695                      700


Ser  Leu  Gln  Ile  Asp  Lys  His  Leu  Gly  Ala  Ala  Asn  Asp  Ala  Ser  Thr
705                      710                      715                      720


Ser  Gln  Val  Glu  Asp  Glu  Ala  Asn  Ser  Tyr  Ala  Lys  Ala  Arg  Asp  Asp
               725                      730                      735


Leu  Val  Lys  Lys  Leu  Pro  Ala  Ser  Tyr  Lys  Thr  Ala  Asp  Pro  Ser  Ser
          740                      745                      750


Ile  Arg  Ala  Ser  Ser  Arg  Pro  Thr  Ile  Phe  Leu  Thr  Gly  Ala  Thr  Gly
          755                      760                      765


Phe  Leu  Gly  Ala  Phe  Leu  Ile  Arg  Asp  Ile  Leu  Gln  Arg  Thr  Ser  Arg
     770                      775                      780


Gln  Leu  Lys  Leu  Ile  Ala  His  Val  Arg  Ala  Lys  Asp  Gln  Lys  Ala  Ala
785                      790                      795                      800


Thr  Glu  Arg  Leu  Thr  Arg  Ser  Leu  Gln  Gly  Tyr  Gly  Ile  Trp  Arg  Asp
               805                      810                      815


Glu  Trp  Ala  Gly  Arg  Leu  Ser  Cys  Val  Val  Gly  Asp  Leu  Ala  Lys  Pro
               820                      825                      830


Gln  Leu  Gly  Ile  Asp  Gln  Pro  Thr  Trp  Glu  Arg  Leu  Ser  Asn  Glu  Val
          835                      840                      845


Asp  Val  Val  Ile  His  Asn  Gly  Ala  Thr  Val  His  Trp  Val  Arg  Arg  Trp
     850                      855                      860


Gln  Asp  Met  Leu  Ala  Ala  Asn  Val  Thr  Ser  Thr  Ile  Glu  Ala  Met  Arg
865                      870                      875                      880


Leu  Cys  Asn  Glu  Gly  Lys  Pro  Lys  Leu  Phe  Thr  Phe  Val  Ser  Ser  Thr
               885                      890                      895
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Ser Val Leu Asp Thr Glu His Tyr Val Gln Leu Ser Glu Arg Gln Leu
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Ser Thr Gly Gln Gly Ala Val Pro Glu Ser Asp Asp Leu Glu Gly Ser
        915                     920                 925


Ala Thr Gly Leu Gly Thr Gly Tyr Gly Gln Thr Lys Trp Ile Ser Glu
    930                     935                 940


Gln Leu Val Arg Glu Ala Gly Arg Arg Gly Leu Arg Gly Ser Val Val
945                     950                 955                 960


Arg Pro Gly Tyr Ile Leu Gly Asp Phe Glu Ser Gly Cys Ser Asn Thr
                965                 970                 975


Asp Asp Phe Leu Ile Arg Phe Leu Lys Gly Cys Ile Gln Leu Gly Thr
            980                 985                 990


Arg Pro Arg Ile Leu Asn Thr Val  Asn Ala Val Pro Val  Asn His Val
            995                 1000                1005


Ala Arg  Val Val Val Ala Ala  Gly Leu Asn Pro Val  Pro Val Gln
    1010                1015                1020


Gly Asn  Glu Gly Val His Val  Val His Val Thr Gly  His Pro Arg
    ·1025               1030                1035


Leu Arg  Met Asn Glu Tyr Leu  Ser Leu Leu Glu Phe  Tyr Gly Tyr
    1040                1045                1050


Lys Val  Pro Glu Val Pro Tyr  Asp Ser Trp Lys Glu  Glu Leu Glu
    1055                1060                1065


Gln Tyr  Val Ser Ala Gly Ala  Gly Val Glu Arg Asp  Gln Glu Gln
    1070                1075                1080


His Ala  Leu Leu Pro Leu Phe  His Leu Cys Ile Ser  Asp Leu Pro
    1085                1090                1095


Ala Asn  Thr Lys Ala Pro Glu  Leu Glu Asp Gln Asn  Ala Val Ala
    1100                1105                1110
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13

Val Leu Lys Ala Asp Ala Glu Ala Trp Thr Gly Leu Asp Glu Ser
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Ala Gly Tyr Gly Ile Gly Arg Glu Asp Val Gly Arg Tyr Leu Arg
    1130   1135   1140

Tyr Leu Ala Met Ile Lys Phe Val Pro Trp Pro Thr Ser Arg Gly
    1145   1150   1155

Arg Pro Leu Pro Glu Val Ser Ile Ser Thr Glu Gln Val Ala Ala
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Met Gly Ala Gly Val Gly Gly Arg Gly Gly Ala Gly Ala Gly Gln
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aacgtgaaca acttcaagat cattgagagc accctgcgag agggcgagca gttcgccaat   240

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cgcagcaacc tggttgacct gctgagcatc tactcgaccg tcgacaagat tggtgtcaac   660

cgtgtcggta ttgctgatac cgtcggttgc gcctcgcccc gccaggtcta cgacctggtc   720

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accaccaaa atg tgc cca tcc tgc gag cct gag caa gcc gct gcc tcc aat   171
            Met Cys Pro Ser Cys Glu Pro Glu Gln Ala Ala Ala Ser Asn
             1   5   10

ggc aat gcg aac ggc aat ggc gcc tcc aat ggc aat gga aac cac gac   219
Gly Asn Ala Asn Gly Asn Gly Ala Ser Asn Gly Asn Gly Asn His Asp

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         15                    20                     25                     30
gga atg act ggt att gag act cgc caa gca caa aac gca cgc tac cag    267
Gly Met Thr Gly Ile Glu Thr Arg Gln Ala Gln Asn Ala Arg Tyr Gln
                     35                  40                  45

cca tca cgg aat ccc tac cag ccc gtc ggt gac ttt ttg tcc aac gtg    315
Pro Ser Arg Asn Pro Tyr Gln Pro Val Gly Asp Phe Leu Ser Asn Val
                 50                  55                  60

aac aac ttc aag atc att gag agc acc ctg cga gag ggc gag cag ttc    363
Asn Asn Phe Lys Ile Ile Glu Ser Thr Leu Arg Glu Gly Glu Gln Phe
             65                  70                  75

gcc aat gcc ttc ttc gac acg gtgagtcaag ccacatcgca agcaaatact       414
Ala Asn Ala Phe Phe Asp Thr
         80                  85

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tttaacctcc gcgcag gcc aag aag att gag atc gcc aag gcg ctg gac gac    586
              Ala Lys Lys Ile Glu Ile Ala Lys Ala Leu Asp Asp
                              90                  95

ttt ggc gtc gac tac atc gag ctc acc agc ccg gct gcc tcg gag cag    634
Phe Gly Val Asp Tyr Ile Glu Leu Thr Ser Pro Ala Ala Ser Glu Gln
             100                 105                 110

tcc agg ctt gac tgc gct gcc atc tgc aag ctg gga ctc aag gcc aag    682
Ser Arg Leu Asp Cys Ala Ala Ile Cys Lys Leu Gly Leu Lys Ala Lys
         115                 120                 125

atc ctc acc cac atc agg tgc cac atg gac gac gcg cgc atc gcc gtc    730
Ile Leu Thr His Ile Arg Cys His Met Asp Asp Ala Arg Ile Ala Val
130                 135                 140                 145

gag acc ggt gtt gac ggc gtc gac att gtc atc ggc acc tct tcg ttc    778
Glu Thr Gly Val Asp Gly Val Asp Ile Val Ile Gly Thr Ser Ser Phe
                 150                 155                 160

ctc atg gag cac tcg cac ggc aag gac atg acc tac atc acc aac acg    826
Leu Met Glu His Ser His Gly Lys Asp Met Thr Tyr Ile Thr Asn Thr
                 165                 170                 175

gcc att gag gtc atc aac ttt gtc aag agc aag ggc atc gag gtc cgc    874
Ala Ile Glu Val Ile Asn Phe Val Lys Ser Lys Gly Ile Glu Val Arg
             180                 185                 190

ttc tca tcc gag gac tcg ttc cgc agc aac ctg gtt gac ctg ctg agc    922
Phe Ser Ser Glu Asp Ser Phe Arg Ser Asn Leu Val Asp Leu Leu Ser
         195                 200                 205

atc tac tcg acc gtc gac aag att ggt gtc aac cgt gtc ggt att gct    970
Ile Tyr Ser Thr Val Asp Lys Ile Gly Val Asn Arg Val Gly Ile Ala
210                 215                 220                 225
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17

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            230                     235                 240

acc ctg cgt ggt gtt gtc tct tg gtgagccaca ggtctgatga atcttgtgct        1071
Thr Leu Arg Gly Val Val Ser Cys
            245

gcttggtgct gatgctaaca gttcgatag t gac att gag aca cac ttc cac aac      1125
                                   Asp Ile Glu Thr His Phe His Asn
                                   250                     255

gac act ggc tgt gcc atc tca aat gct ttt tgc gct ttg gag gcc ggt        1173
Asp Thr Gly Cys Ala Ile Ser Asn Ala Phe Cys Ala Leu Glu Ala Gly
            260                     265                 270

gct acc cac atc gac acg tgt gtc ctg ggt atc ggc gag cgt aac gga        1221
Ala Thr His Ile Asp Thr Cys Val Leu Gly Ile Gly Glu Arg Asn Gly
    275                     280                 285

att acc cct ctt gga ggt ctg atg gct cgc atg att gtc ggc tcc aag        1269
Ile Thr Pro Leu Gly Gly Leu Met Ala Arg Met Ile Val Gly Ser Lys
290                     295                 300                 305

gac tac gtt ctg agc aag tac aag ctg cac aag ctc aag gac att gag        1317
Asp Tyr Val Leu Ser Lys Tyr Lys Leu His Lys Leu Lys Asp Ile Glu
            310                     315                 320

gag ctt gtt gcc gac gcc gtt cag gtc aac at gtaagttttg ccatcccagt       1369
Glu Leu Val Ala Asp Ala Val Gln Val Asn Ile
            325                     330

gcagttttca ttctgggtag gattgctaac attttgtctc tgtag t cct ttc aat        1424
                                               Pro Phe Asn
                                               335

aac tac atc act ggt ttc tgt gct ttc acc cac aa gtatgttccg             1469
Asn Tyr Ile Thr Gly Phe Cys Ala Phe Thr His Lys
            340                     345

tcacacactg gtatctacta ttgattcaaa actaactcgt tgctatag g gcc ggt        1524
                                                       Ala Gly

atc cat gcc aag gct att ctc aag aac ccc tca aca tat gag att att       1572
Ile His Ala Lys Ala Ile Leu Lys Asn Pro Ser Thr Tyr Glu Ile Ile
350                     355                 360                 365

gtatgttttt gatctgttca cgcactgtgc cagcatgggt atgatgagcc gaaaatacta      1632

acccttgatt aatcag gac ccg act ctg ttc ggc atc act cgc tat gtc cac     1684
                Asp Pro Thr Leu Phe Gly Ile Thr Arg Tyr Val His
                            370                 375

ttc gcc agc aga ttg acg gga tgg aac gca atc aag agc aga gca tcg        1732
Phe Ala Ser Arg Leu Thr Gly Trp Asn Ala Ile Lys Ser Arg Ala Ser
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              380                    385                     390
cag ctc aac att gag atg acg gat gag cag tgc aag gag tgc act gcc     1780
Gln Leu Asn Ile Glu Met Thr Asp Glu Gln Cys Lys Glu Cys Thr Ala
    395                     400                     405

aag atc aag ctg ttg gct gac att agg ccg atc gct atc gac gac gcc     1828
Lys Ile Lys Leu Leu Ala Asp Ile Arg Pro Ile Ala Ile Asp Asp Ala
410                     415                     420                 425

gac tcc atc att cac gca ttc cac cgc agc atc aac tcg ggc cag cct     1876
Asp Ser Ile Ile His Ala Phe His Arg Ser Ile Asn Ser Gly Gln Pro
                    430                     435                 440

att cag tat ctc gga agc ctg ctc ccc aac atg acg gag gag gag aag     1924
Ile Gln Tyr Leu Gly Ser Leu Leu Pro Asn Met Thr Glu Glu Glu Lys
                445                     450                     455

gcc gcc ctg gca gat gta gag cgc cgt gag tcg aac gat gcc gag caa     1972
Ala Ala Leu Ala Asp Val Glu Arg Arg Glu Ser Asn Asp Ala Glu Gln
                460                     465                     470

ccg gcg gcc aag agg gcc aag gtc gag gct gtt gca t gagcacaacg        2019
Pro Ala Ala Lys Arg Ala Lys Val Glu Ala Val Ala
            475                     480                     485

gaatttttga gcattgtcga agcgtgagcg agtcacatat atattgttga cgcagaattt    2079

tggtggtcaa agggaagtac agaaaggcct tgggctttga ttttcctaac cccaaagcgt    2139

tgacattttt attatgtctt cttctgtctg catacgaagt caaaaaagga aggagaaagg    2199

aaaagtatcg tcaggatggg atggtttacg gatctacatt ggtaccggag ctattcaagg    2259

atagattgtg tttgctttga tttgccccca tggatgagtt ggggcctttt gctgatttgc    2319

tatatgttgc tataccattg aatgaaa                                       2346
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<210> 6

<211> 449

<212> PRT

<213> Magnaporthe grisea

<400> 6

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Met Cys Pro Ser Cys Glu Pro Glu Gln Ala Ala Ala Ser Asn Gly Asn
1               5                   10                  15


Ala Asn Gly Asn Gly Ala Ser Asn Gly Asn Gly Asn His Asp Gly Met
            20                  25                  30
```

19

```
Thr Gly Ile Glu Thr Arg Gln Ala Gln Asn Ala Arg Tyr Gln Pro Ser
        35              40                  45

Arg Asn Pro Tyr Gln Pro Val Gly Asp Phe Leu Ser Asn Val Asn Asn
    50              55                  60

Phe Lys Ile Ile Glu Ser Thr Leu Arg Glu Gly Glu Gln Phe Ala Asn
65              70                  75                  80

Ala Phe Phe Asp Thr Ala Lys Lys Ile Glu Ile Ala Lys Ala Leu Asp
                85                  90                  95

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

Gln Ser Arg Leu Asp Cys Ala Ala Ile Cys Lys Leu Gly Leu Lys Ala
        115                 120                 125

Lys Ile Leu Thr His Ile Arg Cys His Met Asp Asp Ala Arg Ile Ala
    130                 135                 140

Val Glu Thr Gly Val Asp Gly Val Asp Ile Val Ile Gly Thr Ser Ser
145                 150                 155                 160

Phe Leu Met Glu His Ser His Gly Lys Asp Met Thr Tyr Ile Thr Asn
                165                 170                 175

Thr Ala Ile Glu Val Ile Asn Phe Val Lys Ser Lys Gly Ile Glu Val
            180                 185                 190

Arg Phe Ser Ser Glu Asp Ser Phe Arg Ser Asn Leu Val Asp Leu Leu
        195                 200                 205

Ser Ile Tyr Ser Thr Val Asp Lys Ile Gly Val Asn Arg Val Gly Ile
    210                 215                 220

Ala Asp Thr Val Gly Cys Ala Ser Pro Arg Gln Val Tyr Asp Leu Val
225                 230                 235                 240

Lys Thr Leu Arg Gly Val Val Ser Cys Asp Ile Glu Thr His Phe His
                245                 250                 255
```

```
Asn Asp Thr Gly Cys Ala Ile Ser Asn Ala Phe Cys Ala Leu Glu Ala
            260                 265                 270


Gly Ala Thr His Ile Asp Thr Cys Val Leu Gly Ile Gly Glu Arg Asn
            275                 280                 285


Gly Ile Thr Pro Leu Gly Gly Leu Met Ala Arg Met Ile Val Gly Ser
    290                 295                 300


Lys Asp Tyr Val Leu Ser Lys Tyr Lys Leu His Lys Leu Lys Asp Ile
305                 310                 315                 320


Glu Glu Leu Val Ala Asp Ala Val Gln Val Asn Ile Pro Phe Asn Asn
            325                 330                 335


Tyr Ile Thr Gly Phe Cys Ala Phe Thr His Lys Ala Gly Ile His Ala
            340                 345                 350


Lys Ala Ile Leu Lys Asn Pro Ser Thr Tyr Glu Ile Ile Asp Pro Thr
            355                 360                 365


Leu Phe Gly Ile Thr Arg Tyr Val His Phe Ala Ser Arg Leu Thr Gly
    370                 375                 380


Trp Asn Ala Ile Lys Ser Arg Ala Ser Gln Leu Asn Ile Glu Met Thr
385                 390                 395                 400


Asp Glu Gln Cys Lys Glu Cys Thr Ala Lys Ile Lys Leu Leu Ala Asp
            405                 410                 415


Ile Arg Pro Ile Ala Ile Asp Asp Ala Asp Ser Ile Ile His Ala Phe
            420                 425                 430


His Arg Ser Ile Asn Ser Gly Gln Pro Ile Gln Ser Leu Gly Ser Leu
            435                 440                 445


Leu
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