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(71) PARADIGM GENETICS, INC.,  
108 Alexander Drive, P.O. Box 14528, RESEARCH TRIANGLE PARK, XX (US).

(72) FRANK, SHERYL (US).  
DARVEAUX, BLAISE (US).  
SKALCHUNES, AMY (US).  
HEINIGER, RYAN (US).

TANZER, MATTHEW (US).  
TARPEY, REX (US).  
PAN, HUAQUIN (US).  
SHUSTER, JEFFREY (US).  
HAMER, LISBETH (US).  
MAHANTY, SANJOY (US).  
LO, SZE-CHUNG (CN).  
ADACHI, KIICHI (US).  
DEZWAAN, TODD (US).  
MONTENEGRO-CHAMORRO, MARIA VICTORIA (US).

(74) SMART & BIGGAR

(54) PROCÉDES D'IDENTIFICATION D'INHIBITEURS D'ALPHA-AMINOADIPATE REDUCTASE ET D'HOMOCITRATE SYNTHASE EN TANT QU'ANTIBIOTIQUES

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

(57)

The present inventors have discovered that .alpha.-Aminoadipate Reductase and Homocitrate Synthase are each essential for fungal pathogenicity. Specifically, the inhibition of .alpha.-Aminoadipate Reductase or Homocitrate Synthase gene expression in fungi results in no signs of successful infection or lesions. Thus, .alpha.-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 .alpha.-Aminoadipate Reductase or Homocitrate Synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably antifungals.



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(54) **Titre : PROCÉDES D'IDENTIFICATION D'INHIBITEURS D'ALPHA-AMINOADIPATE REDUCTASE ET D'HOMOCITRATE SYNTHASE EN TANT QU'ANTIBIOTIQUES**

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

(57) **Abrégé/Abstract:**

The present inventors have discovered that  $\alpha$ -Amino adipate Reductase and Homocitrate Synthase are each essential for fungal pathogenicity. Specifically, the inhibition of  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase gene expression in fungi results in no signs of successful infection or lesions. Thus,  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably antifungals.



(72) **Inventeurs(suite)/Inventors(continued):** LO, SZE-CHUNG, CN; MONTENEGRO-CHAMORRO, MARIA VICTORIA, US; FRANK, SHERYL, US; DARVEAUX, BLAISE, US; MAHANTY, SANJOY, US; HEINIGER, RYAN, US; SKALCHUNES, AMY, US; PAN, HUAQUIN, US; TARPEY, REX, US

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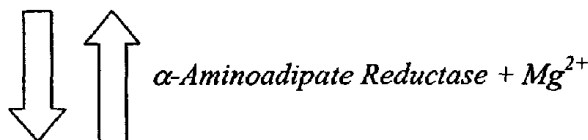
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- (74) Agent: **SPENCER, Deborah**; Paradigm Genetics, Inc., 108 Alexander Drive, P.O. Box 14528, Research Triangle Park, NC 27709-4528 (US).
- (71) Applicant (for all designated States except US): **PARADIGM GENETICS, INC.** [US/US]; 108 Alexander 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**
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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

L-2-Aminoadipate 6-semialdehyde + NADP<sup>+</sup> + AMP + pyrophosphate + H<sub>2</sub>O

(57) Abstract: The present inventors have discovered that  $\alpha$ -Aminoadipate Reductase and Homocitrate Synthase are each essential for fungal pathogenicity. Specifically, the inhibition of  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase gene expression in fungi results in no signs of successful infection or lesions. Thus,  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase expression or activity. The methods of the invention are useful for the identification of antibiotics, preferably antifungals.

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METHODS FOR THE IDENTIFICATION OF INHIBITORS OF  
ALPHA-AMINOADIPATE REDUCTASE AND HOMOCITRATE SYNTHASE AS  
ANTIBIOTICS

5

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*,  
*Apiognomonina*, *Apiosporina*, *Armillaria*, *Ascochyta*, *Aspergillus*, *Bipolaris*,

*Bjerkandera, Botryosphaeria, Botrytis, Ceratobasidium, Ceratocystis, Cercospora,*  
*Cercosporidium, Cerotelium, Cerrena, Chondrostereum, Chryphonectria,*  
*Chrysomyxa, Cladosporium, Claviceps, Cochliobolus, Coleosporium,*  
*Colletotrichum, 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,*  
*Linosporea, 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, Microsporium, Trichophyton,*

*Epidermophyton*, *Candida*, *Histoplasma*, *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  
5 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  
10 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*  
15 *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*  
20 *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  
25 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.  
30 (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 al.* 15 *et 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 gene, gene product, or biochemical pathway, and are thus candidates for antifungal, biocide, and biostatic materials.

### SUMMARY OF THE INVENTION

Surprisingly, the present inventors have discovered that *in vivo* disruption of the gene encoding  $\alpha$ -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  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase or, separately, of Homocitrate Synthase. The methods of the invention are useful for the identification of antibiotics, preferably fungicides.

### BRIEF DESCRIPTION OF THE FIGURES

Figure 1 shows the reaction performed by  $\alpha$ -Aminoadipate Reductase (AAR1) reaction. The Substrates/Products are L-2-Aminoadipate + NADPH + ATP and the Products/Substrates are L-2-Aminoadipate 6-semialdehyde + NADP<sup>+</sup> + AMP + pyrophosphate + H<sub>2</sub>O. The function of the  $\alpha$ -Aminoadipate Reductase enzyme is the interconversion of L-2-Aminoadipate, NADPH, and ATP to L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O. This reaction is part of the 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<sub>2</sub>O + 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<sub>2</sub>O 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 expression product in a deleterious manner. Thus, the direct target of the compound may be, for example, at an upstream component which reduces transcription from the gene, resulting in a lower level of expression. Likewise, the compound may affect the level of translation of a polypeptide expression product, or may act on a downstream component of a biochemical pathway in which the expression product of the gene has a major biological role. Consequently, such a compound can be said to be active against the gene, against the gene product, or against the related component either upstream or downstream of that gene or expression product. While the term "active against" encompasses a broad range of potential activities, it also implies some degree of specificity of target. Therefore, for example, a general protease is not "active against" a particular gene which produces a polypeptide product. In contrast, a compound which inhibits a particular enzyme is active against that enzyme and against the gene which codes for that enzyme.

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

As used herein, the term " $\alpha$ -Amino adipate Reductase (EC 1.2.1.31),  $\alpha$ -Amino adipate Reductase polypeptide,  $\alpha$ -Amino adipate semialdehyde dehydrogenase, or Alpha-Amino adipate 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-Amino adipate, NADPH, and ATP with L-2-Amino adipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O.

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, preferably non-covalent, that holds two molecules together. For example, two such molecules could be an enzyme and an inhibitor of that enzyme. Non-covalent interactions include hydrogen bonding, ionic interactions among charged groups, van

der Waals interactions and hydrophobic interactions among nonpolar groups. One or more of these interactions can mediate the binding of two molecules to each other.

The term "biochemical pathway" or "pathway" refers to a connected series of biochemical reactions normally occurring in a cell, or more broadly a cellular event such as cellular division or DNA replication. Typically, the steps in such a biochemical pathway act in a coordinated fashion to produce a specific product or products or to produce some other particular biochemical action. Such a biochemical pathway requires the expression product of a gene if the absence of that expression product either directly or indirectly prevents the completion of one or more steps in that pathway, thereby preventing or significantly reducing the production of one or more normal products or effects of that pathway. Thus, an agent specifically inhibits such a biochemical pathway requiring the expression product of a particular gene if the presence of the agent stops or substantially reduces the completion of the series of steps in that pathway. Such an agent, may, but does not necessarily, act directly on the expression product of that particular gene.

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

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

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

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

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

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

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

"Fungi" (singular: fungus) refers to whole fungi, fungal organs and tissues (e.g., asci, hyphae, pseudohyphae, rhizoid, sclerotia, sterigmata, spores, sporodochia, sporangia, synnemata, conidia, ascostroma, cleistothecia, mycelia, perithecia, basidia

and the like), spores, fungal cells and the progeny thereof. Fungi are a group of organisms (about 50,000 known species), including, but not limited to, mushrooms, mildews, moulds, yeasts, *etc.*, comprising the kingdom Fungi. They can either exist as single cells or make up a multicellular body called a mycelium, which consists of  
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 "H<sub>2</sub>O" means water.

As used herein, the term "AAR1" means a gene encoding  $\alpha$ -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<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O.

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*  $\alpha$ -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, H<sub>2</sub>O, 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 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<sub>2</sub>O, and 2-oxoglutarate with 2-hydroxybutane-1,2,4-tricarboxylate and CoA.

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

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

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

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

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

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

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

The term "inhibitor", as used herein, refers to a chemical substance that inactivates the enzymatic activity of  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase, or substantially reduces the level of enzymatic activity, wherein "substantially" means a reduction at least as great as the standard deviation for a measurement, preferably a reduction by 50%, more preferably a reduction of at least one magnitude, i.e. to 10%. The inhibitor may function by interacting directly with



the enzyme, a cofactor of the enzyme, the substrate of the enzyme, or any combination thereof.

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

As used herein, the term "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 either the BLAST program (Basic Local Alignment Search Tool; (Altschul, S.F., W. Gish, *et al.* (1990) *J Mol Biol* 215: 403 - 10 (PMID: 2231712)) at the National Center for Biotechnology or using Smith Waterman Alignment (Smith, T. F. and M. S. Waterman (1981) *J Mol Biol* 147: 195 - 7 (PMID: 7265238)) as incorporated into GeneMatcher Plus<sup>TM</sup>. 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 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  $\alpha$ -Aminoadipate Reductase and a molecule or compound, wherein the interaction is dependent upon the primary amino acid sequence and/or the conformation of  $\alpha$ -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  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate 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)  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase protein may have the amino acid sequence of a naturally occurring  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase is a fungal  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase. The cDNA (SEQ ID NO: 1) encoding the  $\alpha$ -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  $\alpha$ -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<sub>2</sub>O. The  $\alpha$ -Aminoadipate Reductase may be from any of the fungi, including ascomycota, zygomycota, basidiomycota, chytridiomycota, and lichens.

By "fungal Homocitrate Synthase" is meant an enzyme that can be found in at  
 25 least one fungus, and which catalyzes the interconversion of acetyl-CoA and H<sub>2</sub>O 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.

In one embodiment, the  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase  
 30 is a *Magnaporthe*  $\alpha$ -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*  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase is from *Magnaporthe grisea*.

5 In various embodiments, the  $\alpha$ -Amino adipate 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 zea-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  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase. The fragments comprise at least 10 consecutive amino acids of an  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase. Preferably, the fragment comprises at least 15, 20, 25, 30, 35,  
30 40, 50, 60, 70, 80, 90, 100, 110, 120, 130, 140, 150, 160, 170, 180, 190, 200, 210, 220, 230, 240, 250, 260, 270, 280, 290, 300, 310, 320, 330, 340, 350, 360, 370, 380, 390, 400, 410, 420, 430, 440, 450, 460, 470, 480, 490, 500, 510, 520, 530, 540, 550, 560, 570, 580, 590, 600, 610, 620, 630, 640, 650, 660, 670, 680, 690, 700, 710, 720, 730, 740, 750, 760, 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  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase. In one embodiment, the fragment is from a *Magnaporthe*  $\alpha$ -  
5 Aminoadipate Reductase or Homocitrate Synthase. Preferably, the fragment contains an amino acid sequence conserved among fungal  $\alpha$ -Aminoadipate Reductases or Homocitrate Synthases.

Polypeptides having at least 50% sequence identity with a fungal  $\alpha$ -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  $\alpha$ -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  $\alpha$ -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*  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50% sequence identity with a fungal  $\alpha$ -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 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 the methods of the invention. For example, the ligand and target are combined in a buffer. Many methods for detecting the binding of a ligand to its target are known in the art, and include, but are not limited to the detection of an immobilized ligand-target complex or the detection of a change in the properties of a target when it is bound to a ligand. For example, in one embodiment, an array of immobilized candidate ligands is provided. The immobilized ligands are contacted with an  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase protein or a fragment or variant thereof, the unbound protein is removed and the bound  $\alpha$ -Amino adipate Reductase is detected. In a preferred embodiment, bound  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase is detected using a labeled binding partner, such as a labeled antibody. In a variation of this assay,  $\alpha$ -Amino adipate 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.

Once a compound is identified as a candidate for an antibiotic, it can be tested for the ability to inhibit  $\alpha$ -Amino adipate 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 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  $\alpha$ -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.  $\alpha$ -Aminoadipate Reductase catalyzes the irreversible or reversible reaction L-2-Aminoadipate and NADPH and ATP = L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O (see Figure 1). Methods for detection of L-2-Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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  $\alpha$ -Aminoadipate Reductase ;
- b) contacting L-2-Aminoadipate and NADPH and ATP with  $\alpha$ -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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O with an  $\alpha$ -Aminoadipate Reductase ;

b) contacting L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O with an  $\alpha$ -Aminoadipate Reductase 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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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 H<sub>2</sub>O 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 H<sub>2</sub>O and 2-oxoglutarate with a homocitrate synthase;

b) contacting acetyl-CoA and H<sub>2</sub>O 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 H<sub>2</sub>O.

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 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<sub>2</sub>O.

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  $\alpha$ -Aminoadipate Reductase or 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  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase may be used in the methods of the invention. Most preferably, the polypeptide has at least 50% sequence identity with a fungal  $\alpha$ -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 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  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50% sequence identity with an  $\alpha$ -Aminoadipate Reductase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an  $\alpha$ -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-Aminoadipate, L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with an  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50% sequence identity with an  $\alpha$ -Aminoadipate Reductase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an  $\alpha$ -Aminoadipate Reductase
- b) contacting L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O, 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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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<sub>2</sub>O 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 activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of a homocitrate synthase
- b) contacting acetyl-CoA and H<sub>2</sub>O 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<sub>2</sub>O.

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 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
- 10 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<sub>2</sub>O.

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,  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase protein and derivatives thereof may be purified from a fungus or may be recombinantly produced in and purified from an archaeal, 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  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate 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:

- 25 a) measuring the expression of an  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase in said cell, cells, tissue, or organism;
- 30

c) comparing the expression of  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase can be measured by detecting the AAR1 or HCS1 primary transcript or mRNA,  $\alpha$ -Amino adipate Reductase or Homocitrate Synthase polypeptide, or  $\alpha$ -Amino adipate 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 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 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 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 be used to control fungal growth. Diseases such as rusts, mildews, and blights spread rapidly once established. Fungicides are thus routinely applied to growing and stored crops as a preventive measure, generally as foliar sprays or seed dressings. For example, compounds that inhibit fungal growth can be applied to a fungus or expressed in a fungus, in order to prevent fungal growth. Thus, the invention provides a method for inhibiting fungal growth, comprising contacting a fungus with a compound identified by the methods of the invention as having antifungal activity.

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

Examples of undesired fungi include, but are not limited to Powdery Scab  
 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., *Fusarium* 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  
5 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:

- 10 a) providing cells having one form of an  $\alpha$ -Aminoadipate Reductase gene, and providing comparison cells having a different form of an  $\alpha$ -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,
- 15 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:

- 20 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,
- 25 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  
30 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 pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics as inhibitors of the substrates, products and enzymes of the pathway. Pathways known in the art may be found at the Kyoto Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers).

Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which 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 accommodates multiple different assays to characterize various compounds, concentrations of compounds, and fungal strains in varying combinations and formats. Certain testing parameters for the screening method can significantly affect the identification of growth inhibitors, and thus can be manipulated to optimize screening efficiency and/or reliability. Notable among these factors are variable sensitivities of different mutants, increasing hypersensitivity with increasingly less permissive conditions, an apparent increase in hypersensitivity with increasing compound concentration, and other factors known to those in the art.

Conditional lethal mutants may identify particular biochemical and/or genetic pathways given that at least one identified target gene is present in that pathway. Knowledge of these pathways allows for the screening of test compounds as candidates for antibiotics. Pathways known in the art may be found at the Kyoto

Encyclopedia of Genes and Genomes and in standard biochemistry texts (Lehninger, A., D. Nelson, et al. (1993) Principles of Biochemistry. New York, Worth Publishers). Thus, in one embodiment, the invention provides a method for screening for test compounds acting against the biochemical and/or genetic pathway or pathways in which 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;
- (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 organism in the paired media in the absence of any test compounds may be performed to control for any inherent differences in growth as a result of the different media. Growth and/or proliferation of an organism is measured by methods well known in the art such as optical density measurements, and the like. In a preferred embodiment, the organism is *Magnaporthe grisea*.

## EXPERIMENTAL

### Example 1

Construction of Plasmids with a Transposon Containing a Selectable Marker.

Construction of Sif transposon: Sif was constructed using the GPS3 vector from the GPS-M mutagenesis system from New England Biolabs, Inc. (Beverly, MA) as a backbone. This system is based on the bacterial transposon Tn7. The following manipulations were done to GPS3 according to Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press. The kanamycin resistance gene (npt) contained between the Tn7 arms was removed by EcoRV digestion. The bacterial hygromycin B phosphotransferase (hph) gene (Gritz and Davies (1983) *Gene* 25: 179 – 88 (PMID: 6319235)) under control of the *Aspergillus nidulans* trpC promoter and terminator (Mullaney *et al.* (1985) *Mol Gen Genet* 199:

37 – 45 (PMID: 3158796)) was cloned by a HpaI/EcoRV blunt ligation into the Tn7 arms of the GPS3 vector yielding pSif1. Excision of the ampicillin resistance gene (bla) from pSif1 was achieved by cutting pSif1 with XmnI and BglII followed by a T4 DNA polymerase treatment to remove the 3' overhangs left by the BglII 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) Molecular Cloning, a Laboratory Manual, 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) Molecular Cloning, a Laboratory Manual, 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  $\mu$ g 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.).

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## Example 4

High Throughput Preparation and Verification of Insertion of  
Transposon into Fungal Genes

*E. coli* strains containing cosmids with transposon insertions were picked to 96  
10 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*

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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 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<sub>2</sub>O and digested with 4 mg/ml beta-glucanase (InterSpex) for 4-6 hours to generate protoplasts. Protoplasts were collected by centrifugation and resuspended in 20% sucrose at the concentration of 2x10<sup>8</sup> protoplasts/ml. 50ul protoplast suspension was mixed with 10-20ug of the cosmid DNA and pulsed using Gene Pulser II (BioRad) set with the following parameters: resistance 200 ohm, capacitance 25uF, voltage 0.6kV. Transformed protoplasts were regenerated in complete agar media (CM, Talbot *et al.* (1993) Plant Cell 5: 1575 - 1590 (PMID: 8312740)) with the addition of 20% sucrose for one day, then overlaid with CM agar media containing hygromycin B (250ug/ml) to select transformants. Transformants were screened for homologous recombination events in the target gene by PCR (Hamer *et al.* (2001) Proc Natl Acad Sci USA 98: 5110 - 15 (PMID: 11296265)). Two independent strains were identified for AAR1 and are hereby referred to as KO1-1 and KO1-11, respectively. For HCS1, two independent strains were also identified and are hereby referred to as HCS1 KO1-1 and KO1-2, respectively.

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#### Example 6

Effect of AAR1 Transposon Insertion on *Magnaporthe* pathogenicity

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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 ( $5 \times 10^4$  conidia per ml in 0.01% Tween-20 (Polyoxyethylensorbitan monolaureate) solution). The inoculated plants were incubated in a dew chamber at 27°C in the dark for 36 hours, and transferred to a growth chamber (27°C 12 hours/21°C 12 hours 70% humidity) for an additional 5.5 days. Leaf samples were taken at 3, 5, and 7 days post-inoculation and examined for signs of successful infection (*i.e.* lesions). Figure 2 shows the effects of AAR1 gene 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% Phytigel, 0.03% Pluronic F68, 1% glucose, 23.5mM NaNO<sub>3</sub>, 20 6.7mM KCl, 3.5mM Na<sub>2</sub>SO<sub>4</sub>, 11mM KH<sub>2</sub>PO<sub>4</sub>, 0.01% *p*-iodonitrotetrazolium violet, 0.1mM MgCl<sub>2</sub>, 1.0mM CaCl<sub>2</sub> and trace elements. Final concentrations of trace elements are: 7.6μM ZnCl<sub>2</sub>, 2.5μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.8μM FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.71μM CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.64μM CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.62μM Na<sub>2</sub>MoO<sub>4</sub>, 18μM H<sub>3</sub>BO<sub>3</sub>. pH adjusted to 6.0 with NaOH. Spores for each strain were harvested into the inoculating fluid. The spore concentrations were adjusted to  $2 \times 10^5$  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<sub>490</sub> measures the extent of tetrazolium dye reduction and the level of growth, and OD<sub>750</sub> measures growth only. Turbidity = OD<sub>490</sub> + OD<sub>750</sub>. 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, HCS1 KO1-1 and KO1-2, obtained in Example 5  
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  
10 for whole plant inoculations. Two-week-old seedlings of cultivar CO39 were sprayed  
with 12 ml of conidial suspension ( $5 \times 10^4$  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  
15 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

## 20 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  
25 tests for the auxotrophic requirement for 94 different metabolites. The inoculating  
fluid consists of 0.05% Phytigel, 0.03% Pluronic F68, 1% glucose, 23.5mM NaNO<sub>3</sub>,  
6.7mM KCl, 3.5mM Na<sub>2</sub>SO<sub>4</sub>, 11mM KH<sub>2</sub>PO<sub>4</sub>, 0.01% *p*-iodonitrotetrazolium violet,  
0.1mM MgCl<sub>2</sub>, 1.0mM CaCl<sub>2</sub> and trace elements. Final concentrations of trace  
elements are: 7.6μM ZnCl<sub>2</sub>, 2.5μM MnCl<sub>2</sub>·4H<sub>2</sub>O, 1.8μM FeCl<sub>2</sub>·4H<sub>2</sub>O, 0.71μM  
30 CoCl<sub>2</sub>·6H<sub>2</sub>O, 0.64μM CuCl<sub>2</sub>·2H<sub>2</sub>O, 0.62μM Na<sub>2</sub>MoO<sub>4</sub>, 18μM H<sub>3</sub>BO<sub>3</sub>. pH adjusted to  
6.0 with NaOH. Spores for each strain were harvested into the inoculating fluid. The  
spore concentrations were adjusted to  $2 \times 10^5$  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<sub>490</sub> measures the extent of tetrazolium dye reduction and the level of growth, and OD<sub>750</sub> measures growth only. Turbidity = OD<sub>490</sub> + OD<sub>750</sub>. Data confirming the annotated gene function is presented as a graph of Turbidity vs. Time showing both the mutant fungi and the wild-type control in the absence (Figure 6A) and presence (Figure 6B) of L-lysine.

#### Example 10

Cloning and Expression Strategies, Extraction and Purification of  $\alpha$ -Aminoacidipate Reductase or Homocitrate Synthase protein.

The following protocol may be employed to obtain a purified  $\alpha$ -Aminoacidipate Reductase protein.

##### **Cloning and expression strategies:**

An AAR1 or HCS1 cDNA gene can be cloned into *E. coli* (pET vectors- Novagen), Baculovirus (Pharming) and Yeast (Invitrogen) expression vectors containing His/fusion protein tags. Evaluate the expression of recombinant protein by SDS-PAGE and Western blot analysis.

##### **Extraction:**

Extract recombinant protein from 250 ml cell pellet in 3 ml of extraction buffer

By sonicating 6 times, with 6 sec pulses at 4°C. Centrifuge extract at 15000xg for 10 min and collect supernatant. Assess biological activity of the recombinant protein by activity assay.

##### **Purification:**

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

Purification protocol: perform all steps at 4°C:

- Use 3 ml Ni-beads (Qiagen)
- Equilibrate column with the buffer
- Load protein extract
- Wash with the equilibration buffer



- Elute bound protein with 0.5 M imidazole

### Example 11

#### Assays for Testing Binding of Test Compounds to $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase

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

- Purified full-length  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase polypeptide with a His/fusion protein tag (Example 10) is bound to a HisGrab<sup>TM</sup> 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 <sup>14</sup>C- $\alpha$ -Aminoadipic acid (Sigma-Aldrich Co.) to the bound AAR1, or for binding of radiolabeled (acetyl-1-<sup>14</sup>C)-coenzyme A (Sigma-Aldrich Co.) to the bound HCS1.
- Screening of test compounds is performed by adding test compound and <sup>14</sup>C- $\alpha$ -Aminoadipic acid (Sigma-Aldrich Co.) to the wells of the HisGrab<sup>TM</sup> plate containing bound AAR1, or performed by adding test compound and (acetyl-1-<sup>14</sup>C)-coenzyme A (Sigma-Aldrich Co.) to the wells of the HisGrab<sup>TM</sup> 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*  $\alpha$ -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.

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  $2 \times 10^5$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100  $\mu\text{g/ml}$ . Solvent only is added to the second culture. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The growth curves of the solvent control sample and the test compound sample are compared. A test compound is an antibiotic candidate if the growth of the culture containing the test compound is less than the growth of the control culture.

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#### Example 12

##### Assays for Testing Inhibitors or Candidates for Inhibition of $\alpha$ -Amino adipate Reductase or Homocitrate Synthase Activity

The enzymatic activity of  $\alpha$ -Amino adipate 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). 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*  $\alpha$ -Amino adipate 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). A polypeptide comprising 10-50 amino acids is generated by subcloning a portion of the AAR1 or

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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  $2 \times 10^5$  spores/ml and the culture is divided. The test compound is added to one culture to a final concentration of 20-100  $\mu\text{g/ml}$ . 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 $\alpha$ -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  $2 \times 10^5$  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 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.

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#### Example 14

##### *In Vivo* Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase with No Activity

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*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 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  $\mu$ M L-lysine to a concentration of  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  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 200 $\mu$ l. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of a test

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compound on a fungal strain and that on the wild type cells are compared.

Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) *Biotechnology* 26: 177 - 221 (PMID: 7749303)).

#### Example 15

*In Vivo* Cell Based Assay Screening Protocol with a Fungal Strain Containing a Mutant Form of  $\alpha$ -Aminoadipate Reductase or Homocitrate Synthase with Reduced 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 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 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  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  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 200 $\mu$ l. Wells with no test compound present (growth control), and wells without cells are included as controls (negative control). The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild-type cells are compared. Compounds that show differential growth inhibition

between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) *Biotechnology* 26: 177 - 221 (PMID: 7749303)).

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

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## 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 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 art (Sambrook *et al.* (1989) Molecular Cloning, a Laboratory Manual, Cold Spring Harbor Laboratory Press). *Magnaporthe grisea* fungal cells containing a mutant form of are grown under standard fungal growth conditions that are well known and described in the art. *Magnaporthe grisea* spores are harvested from cultures grown on complete agar medium containing 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 to a concentration of  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  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 concentrations). The total volume in each well is 200 $\mu$ l. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. Wild type cells are screened under the same conditions. The effect of each compound on the mutant and wild-type fungal strains is measured against the growth control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of a test compound on a fungal strain and that on the wild type cells are compared. Compounds that show differential growth inhibition between the mutant and the wild type are identified as potential antifungal compounds. Similar protocols may be found in Kirsch and DiDomenico ((1994) *Biotechnology* 26: 177 - 221 (PMID: 7749303)).

## Example 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

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

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- 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 *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 AAR1 gene (see Example 5). Transformants are selected on minimal agar medium lacking L-lysine. Only transformants carrying a functional AAR1 gene will grow.

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Wild-type strains of *Magnaporthe grisea* and strains containing a heterologous form of AAR1 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  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  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 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

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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)).

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#### 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

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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:

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

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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  $2 \times 10^5$  spores per ml. Approximately  $4 \times 10^4$  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 concentrations). The total volume in each well is 200µl. Wells with no test compound present, and wells without cells are included as controls. The plates are incubated at 25°C for seven days and optical density measurements at 590nm are taken daily. The effect of each compound on the wild-type and heterologous fungal strains is measured against the growth control and the percent of inhibition is calculated as the  $OD_{590}$  (fungal strain plus test compound) /  $OD_{590}$  (growth control) x 100. The percent of growth inhibition as a result of a test compound on the wild-type and heterologous fungal strains are compared. Compounds that show differential growth inhibition between the wild-type and heterologous strains are identified as potential antifungal compounds with specificity to the native or heterologous HCS1 gene products. Similar protocols may be found in Kirsch and DiDomenico ((1994) *Biotechnology* 26: 177 - 221 (PMID: 7749303)).

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## 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 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 hemacytometer 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  $2 \times 10^5$  spores per ml. The minimal growth media 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  $4 \times 10^4$  spores/well). For each well containing a spore suspension in minimal media, an additional well is present containing a spore suspension in minimal medium

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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 200 $\mu$ l. 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 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 to exemplify various specific embodiments of the invention and do not limit its scope in any manner.

SEQUENCE LISTING

<110> Tanzer, Matthew M  
Shuster, Jeffrey R  
Hamer, Lisbeth  
Adachi, Kiichi  
DeZwaan, Todd M  
Lo, Sze Chung C  
Montenegro-Chamorro, Maria V  
Frank, Sheryl A  
Darveaux, Blaise A  
Mahanty, Sanjoy K  
Heiniger, Ryan W  
Skalchunes, Amy R  
Pan, Huaqin  
Tarpey, Rex

<120> METHODS FOR THE IDENTIFICATION OF INHIBITORS OF  
ALPHA-AMINOADIPATE REDUCTASE AND HOMOCITRATE SYNTHASE AS ANTIBIOTICS

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Leu



## CLAIMS

What is claimed is:

1. A method for identifying a test compound as a candidate for an antibiotic,  
5 comprising:  
a) contacting an  $\alpha$ -Aminoadipate Reductase polypeptide with said test compound;  
and  
b) detecting the presence or absence of binding between said test compound and  
said  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase polypeptide is a  
fungal  $\alpha$ -Aminoadipate Reductase polypeptide.
- 15 3. The method of claim 1, wherein said  $\alpha$ -Aminoadipate Reductase polypeptide is a  
*Magnaporthe*  $\alpha$ -Aminoadipate Reductase polypeptide.
4. The method of claim 1, wherein said  $\alpha$ -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 of a fungal  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50% sequence identity with a fungal  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase;
  - 20 b) contacting L-2-Aminoadipate and NADPH and ATP with  $\alpha$ -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<sup>+</sup>, NADPH, AMP,
  - 25 ATP, H<sub>2</sub>O 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  $\alpha$ -Aminoadipate Reductase is a fungal  $\alpha$ -  
5 Aminoadipate Reductase.
10. The method of claim 8, wherein said  $\alpha$ -Aminoadipate Reductase is a  
*Magnaporthe*  $\alpha$ -Aminoadipate Reductase.
- 10 11. The method of claim 8, wherein said  $\alpha$ -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<sup>+</sup>, AMP, pyrophosphate,  
and H<sub>2</sub>O with an  $\alpha$ -Aminoadipate Reductase;  
b) contacting L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate,  
and H<sub>2</sub>O with an  $\alpha$ -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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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  $\alpha$ -Aminoadipate Reductase is a fungal  $\alpha$ -Aminoadipate Reductase.

10 15. The method of claim 13, wherein said  $\alpha$ -Aminoadipate Reductase is a *Magnaporthe*  $\alpha$ -Aminoadipate Reductase.

16. The method of claim 13, wherein said  $\alpha$ -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  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50%

sequence identity with an  $\alpha$ -Aminoadipate Reductase and having at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an  $\alpha$ -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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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<sup>+</sup>, AMP, pyrophosphate,  
15 and H<sub>2</sub>O with a polypeptide selected from the group consisting of: a polypeptide having at least 50% sequence identity with an  $\alpha$ -Aminoadipate Reductase, a polypeptide having at least 50% sequence identity with an  $\alpha$ -Aminoadipate Reductase and at least 10% of the activity thereof, and a polypeptide comprising at least 100 consecutive amino acids of an  $\alpha$ -Aminoadipate Reductase

20 b) contacting L-2-Aminoadipate 6-semialdehyde, NADP<sup>+</sup>, AMP, pyrophosphate, and H<sub>2</sub>O, 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<sup>+</sup>, NADPH, AMP, ATP, H<sub>2</sub>O 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  $\alpha$ -Aminoacidipate 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  $\alpha$ -Aminoacidipate Reductase in said fungus or  
10 fungal cell;

c) comparing the expression of  $\alpha$ -Aminoacidipate 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  $\alpha$ -Aminoacidipate Reductase is SEQ ID NO:  
3.

24. The method of claim 20, wherein the expression of  $\alpha$ -Aminoacidipate Reductase is  
25 measured by detecting AAR1 mRNA.

25. The method of claim 20, wherein the expression of  $\alpha$ -Amino adipate Reductase is measured by detecting  $\alpha$ -Amino adipate 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  $\alpha$ -Amino adipate Reductase gene, and providing comparison cells having a different form of an  $\alpha$ -Amino adipate 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;
- 10

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  $\alpha$ -Amino adipate Reductase are fungal  $\alpha$ -Amino adipate Reductases.

20

30. The method of claim 26, wherein at least one form is a *Magnaporthe*  $\alpha$ -Amino adipate Reductase.

31. The method of claim 26 wherein said form and said comparison form of the  $\alpha$ -Aminoadipate Reductase are non-fungal  $\alpha$ -Aminoadipate Reductases.
- 5 32. The method of claim 26 wherein one form of the  $\alpha$ -Aminoadipate Reductase is a fungal  $\alpha$ -Aminoadipate Reductase, and the other form is a non-fungal  $\alpha$ -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 biosynthesis gene.
- 10
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 decrease in growth, viability, or pathogenicity of said fungus or fungal cells.
- 15
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 medium, wherein said second medium contains a higher level of lysine than said first medium;
- 20
- (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: 3.

20

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, comprising:

25

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 candidate by the method of claim 50 has antifungal activity, further comprising:  
15 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<sub>2</sub>O and 2-oxoglutarate with a homocitrate synthase;  
b) contacting acetyl-CoA and H<sub>2</sub>O 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<sub>2</sub>O;  
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<sub>2</sub>O;

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<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O;

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<sub>2</sub>O;

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.

5

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.

10

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.

15

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 biosynthesis gene.

10

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

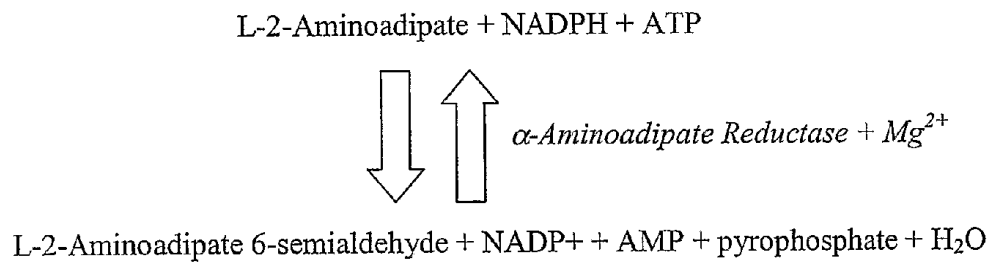
25 NO: 4.

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*

*Figure 2*

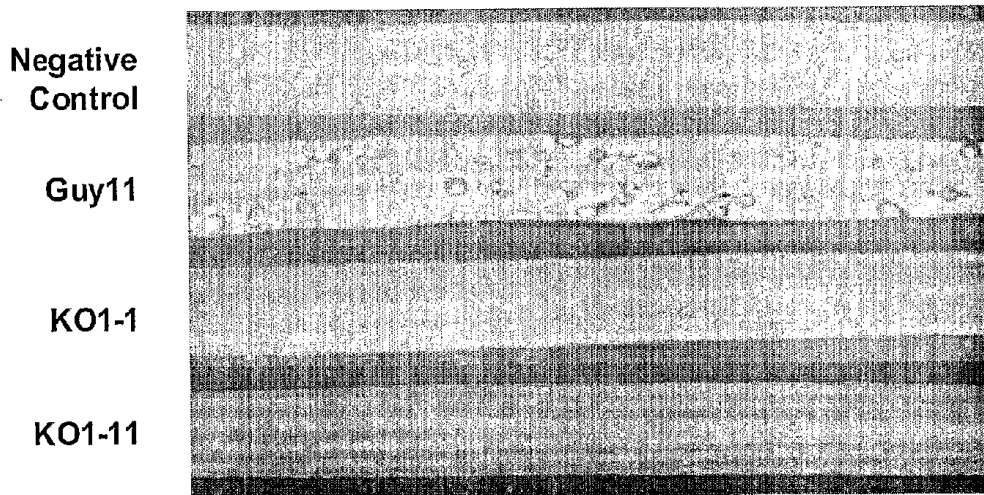
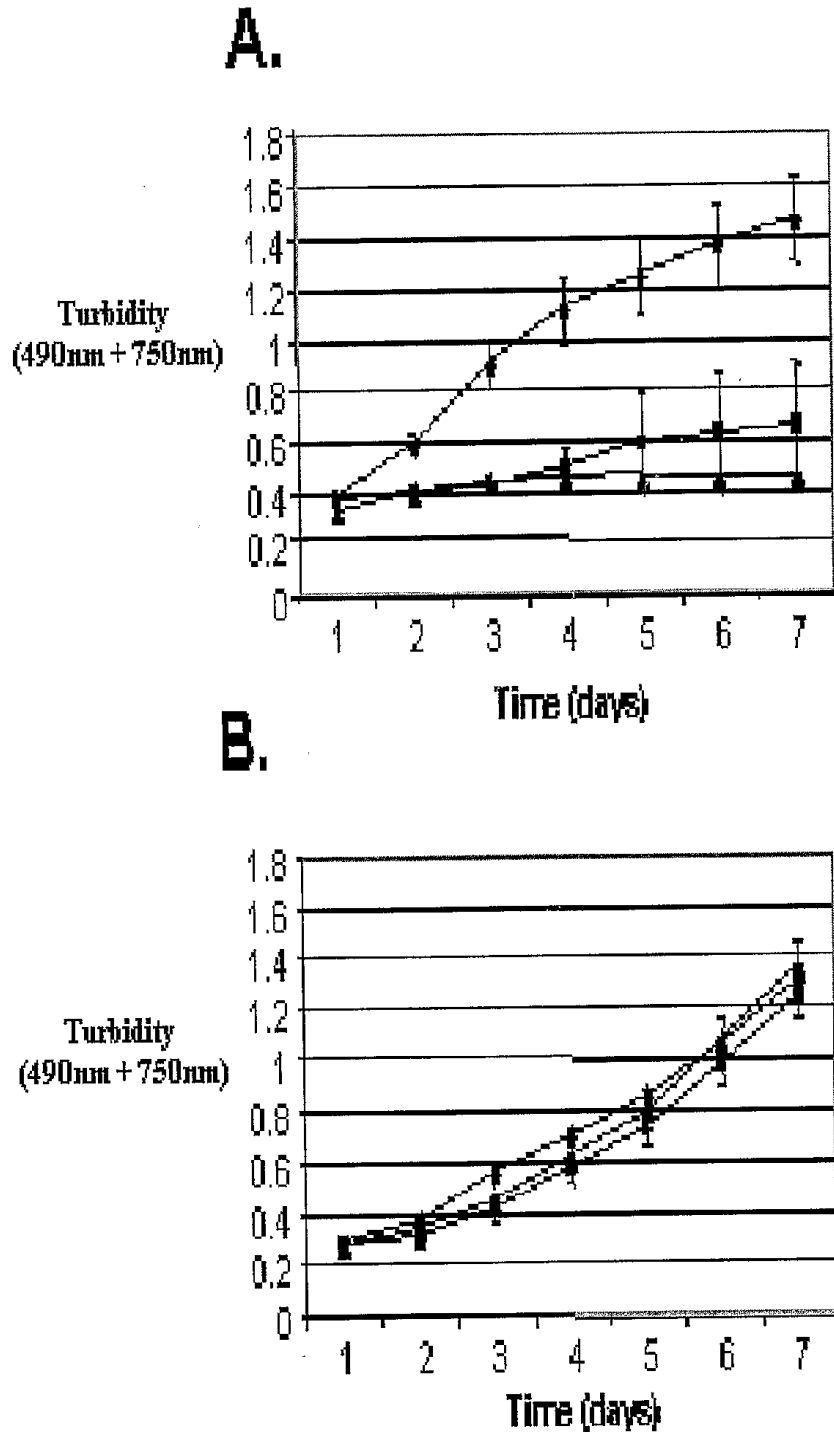
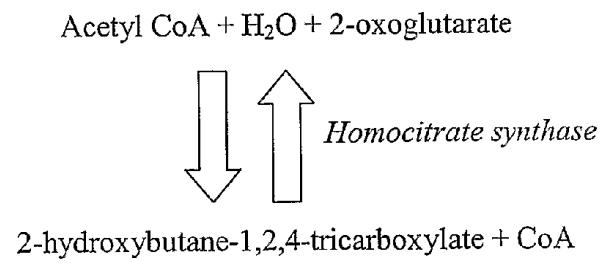


Figure 3



*Figure 4*



*Figure 5*

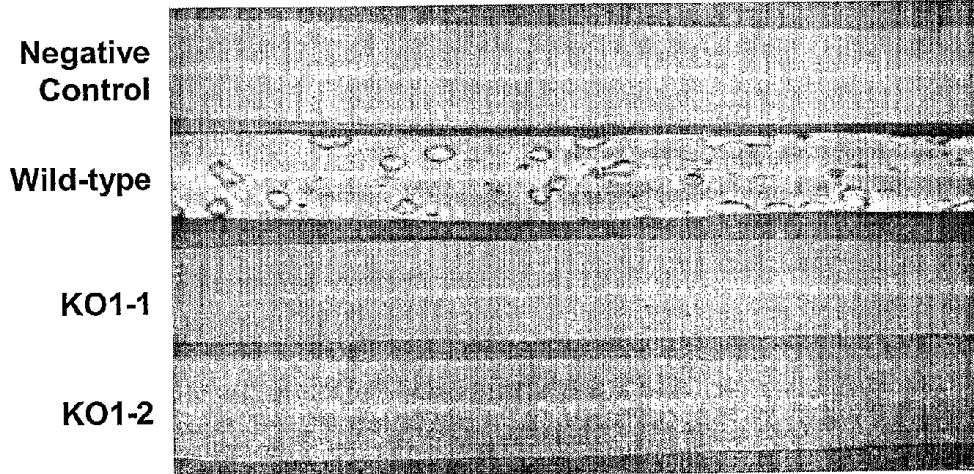
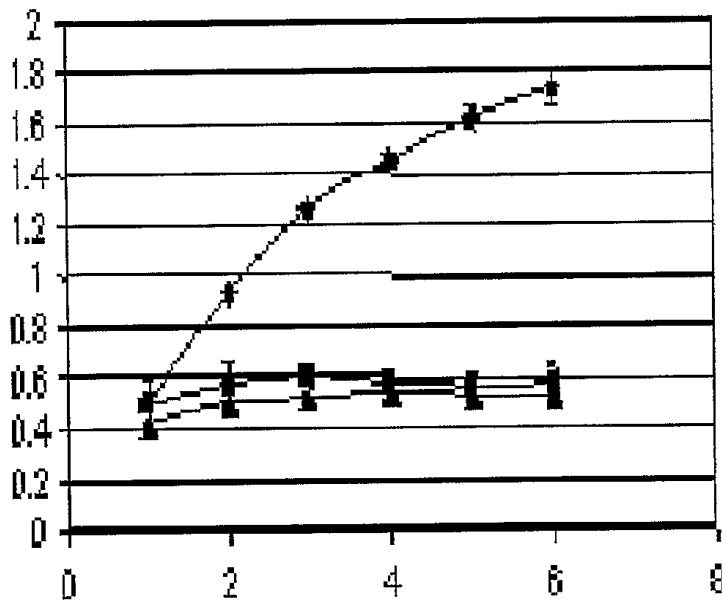


Figure 6

**A.**



**B.**

