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(54) **GENE ATRD DE MULTIRESISTANCE MEDICAMENTEUSE
D'ASPERGILLUS NIDULANS**
(54) **MULTIPLE DRUG RESISTANCE GENE ATRD OF
ASPERGILLUS NIDULANS**

(57) L'invention se rapporte à des composés d'acides nucléiques isolés codant une protéine de multirésistance médicamenteuse d'*Aspergillus nidulans*. L'invention se rapporte également à des vecteurs et à des cellules hôtes transformées comportant l'ADN codant la multirésistance médicamenteuse d'*Aspergillus nidulans*, atrD. Enfin, l'invention se rapporte à des analyses mettant en oeuvre ces cellules hôtes transformées.

(57) The invention provides isolated nucleic acid compounds encoding a multiple drug resistance protein of *Aspergillus nidulans*. Vectors and transformed host cells comprising the multiple drug resistance-encoding DNA of *Aspergillus nidulans* atrD are also provided. The invention further provides assays which utilize these transformed host cells.

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(21) International Application Number: PCT/US98/27300 (22) International Filing Date: 22 December 1998 (22.12.98) (30) Priority Data: 08/996,545 23 December 1997 (23.12.97) US (71) Applicants: ELI LILLY AND COMPANY [US/US]; Lilly Corporate Center, Indianapolis, IN 46285 (US). WAGENINGEN AGRICULTURAL UNIVERSITY [NL/NL]; Bestuurscentrum, Costerweg 50, NL-6701 BH Wageningen (NL). (72) Inventors: ANDRADE, Alan, C.; Bornsesteeg 7, NL-6708 GB Wageningen (NL). DE WAARD, Maarten, A.; Diedenweg 141B, NL-6706 CN Wageningen (NL). PEERY, Robert, Brown; 372 Sycamore Street, Brownsburg, IN 46112 (US). SKATRUD, Paul, Luther; 5233 Tony Drive, Greenwood, IN 46143 (US). (74) Agents: WEBSTER, Thomas, D. et al.; Eli Lilly and Company, Lilly corporate Center, Indianapolis, IN 46285 (US).	(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG). Published <i>With international search report.</i>	
(54) Title: MULTIPLE DRUG RESISTANCE GENE <i>atrD</i> OF <i>ASPERGILLUS NIDULANS</i>		
(57) Abstract The invention provides isolated nucleic acid compounds encoding a multiple drug resistance protein of <i>Aspergillus nidulans</i> . Vectors and transformed host cells comprising the multiple drug resistance-encoding DNA of <i>Aspergillus nidulans atrD</i> are also provided. The invention further provides assays which utilize these transformed host cells.		

MULTIPLE DRUG RESISTANCE GENE *atrD* OF *ASPERGILLUS NIDULANS***Technical Field of the Invention**

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This invention relates to recombinant DNA technology. In particular, the invention concerns the cloning of nucleic acid encoding a multiple drug resistance protein of *Aspergillus nidulans*.

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Background of the Invention

Multiple drug resistance (MDR) mediated by the human *mdr-1* gene product was initially recognized during the course of developing regimens for cancer chemotherapy (Fojo et al., 1987, *Journal of Clinical Oncology* 5:1922-1927). A multiple drug resistant cancer cell line exhibits resistance to high levels of a large variety of cytotoxic compounds. Frequently these cytotoxic compounds will have no common structural features nor will they interact with a common target within the cell. Resistance to these cytotoxic agents is mediated by an outward directed, ATP-dependent pump encoded by the *mdr-1* gene. By this mechanism, toxic levels of a particular cytotoxic compound are not allowed to accumulate within the cell.

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MDR-like genes have been identified in a number of divergent organisms including numerous bacterial species,

the fruit fly *Drosophila melanogaster*, *Plasmodium falciparum*, the yeast *Saccharomyces cerevisiae*, *Caenorhabditis elegans*, *Leishmania donovani*, marine sponges, the plant *Arabidopsis thaliana*, as well as *Homo sapiens*. Extensive searches have revealed several classes of compounds that are able to reverse the MDR phenotype of multiple drug resistant human cancer cell lines rendering them susceptible to the effects of cytotoxic compounds. These compounds, referred to herein as "MDR inhibitors", include for example, calcium channel blockers, anti-arrhythmics, antihypertensives, antibiotics, antihistamines, immuno-suppressants, steroid hormones, modified steroids, lipophilic cations, diterpenes, detergents, antidepressants, and antipsychotics (Gottesman and Pastan, 1993, *Annual Review of Biochemistry* 62:385-427). Clinical application of human MDR inhibitors to cancer chemotherapy has become an area of intensive focus for research.

On another front, the discovery and development of antifungal compounds for specific fungal species has also met with some degree of success. *Candida* species represent the majority of fungal infections, and screens for new antifungal compounds have been designed to discover anti-*Candida* compounds. During development of antifungal agents, activity has generally been optimized based on activity against *Candida albicans*. As a consequence, these anti-*Candida* compounds frequently do not possess clinically significant activity against other fungal species such as *Aspergillus nidulans*. However, it is interesting to note that at higher concentrations some anti-*Candida* compounds are able to kill other fungal species such as *A. nidulans* and *A. fumigatus*. This type of observation suggests that the antifungal target(s) of these anti-*Candida* compounds is present in *A. nidulans* as well. Such results indicate that *A. nidulans* may possess a natural mechanism of resistance that permits them to survive in clinically relevant concentrations of antifungal compounds. Until the present invention, such a general mechanism of resistance to

antifungal compounds in *A. nidulans* has remained undescribed.

Summary of the Invention

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The invention provides, *inter alia*, isolated nucleic acid molecules that comprise nucleic acid encoding a multiple drug resistance protein from *Aspergillus nidulans*, herein referred to as *atrD*, vectors encoding *atrD*, and host
10 cells transformed with these vectors.

In another embodiment, the invention provides a method for determining the fungal MDR inhibition activity of a compound which comprises:

15 a) placing a culture of fungal cells, transformed with a vector capable of expressing *atrD*, in the presence of:

(i) an antifungal agent to which said fungal cell is resistant, but to which said fungal cell is sensitive in its untransformed state;

20 (ii) a compound suspected of possessing fungal MDR inhibition activity; and

b) determining the fungal MDR inhibition activity of said compound by measuring the ability of the antifungal agent to inhibit the growth of said fungal cell.

25 In still another embodiment the present invention relates to strains of *A. nidulans* in which the *atrD* gene is disrupted or otherwise mutated such that the *atrD* protein is not produced in said strains.

30 In yet another embodiment, the present invention relates to a method for identifying new antifungal compounds comprising the use of *atrD* gene disruption or gene replacement strains of *A. nidulans*.

Detailed Description of the Invention

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The present invention provides isolated nucleic acid molecules that comprise a nucleic acid sequence encoding atrD. The cDNA (complementary deoxyribonucleic acid) sequence encoding atrD is provided in the Sequence Listing as SEQ ID NO: 1. The amino acid sequence of the protein encoded by atrD is provided in the Sequence Listing as SEQ ID NO: 2.

Those skilled in the art will recognize that the degenerate nature of the genetic code enables one to construct many different nucleic acid sequences that encode the amino acid sequence of SEQ ID NO: 2. The cDNA sequence depicted by SEQ ID NO: 1 is only one of many possible atrD-encoding sequences. Consequently, the constructions described below and in the accompanying examples for the preferred nucleic acid molecules, vectors, and transformants of the invention are illustrative and are not intended to limit the scope of the invention.

All nucleotide and amino acid abbreviations used in this disclosure are those accepted by the United States Patent and Trademark Office as set forth in 37 C.F.R. §1.822(b) (1994).

The term "vector" refers to any autonomously replicating or integrating agent, including but not limited to plasmids, cosmids, and viruses (including phage), comprising a nucleic acid molecule to which one or more additional nucleic acid molecules can be added. Included in the definition of "vector" is the term "expression vector". Vectors are used either to amplify and/or to express deoxyribonucleic acid (DNA), either genomic or cDNA, or RNA (ribonucleic acid) which encodes atrD, or to amplify DNA or RNA that hybridizes with DNA or RNA encoding atrD.

The term "expression vector" refers to vectors which comprise a transcriptional promoter (hereinafter "promoter") and other regulatory sequences positioned to drive expression of a DNA segment that encodes atrD. Expression vectors of the present invention are replicable DNA

constructs in which a DNA sequence encoding atrD is operably linked to suitable control sequences capable of effecting the expression of atrD in a suitable host. Such control sequences include a promoter, an optional operator sequence to control transcription, a sequence encoding suitable mRNA ribosomal binding sites, and sequences which control termination of transcription and translation. DNA regions are operably linked when they are functionally related to each other. For example, a promoter is operably linked to a DNA coding sequence if it controls the transcription of the sequence, or a ribosome binding site is operably linked to a coding sequence if it is positioned so as to permit translation.

The term "MDR inhibition activity" refers to the ability of a compound to inhibit the MDR activity of a host cell, thereby increasing the antifungal activity of an antifungal compound against said host cell.

In the present invention, atrD may be synthesized by host cells transformed with vectors that provide for the expression of DNA encoding atrD. The DNA encoding atrD may be the natural sequence or a synthetic sequence or a combination of both ("semi-synthetic sequence"). The *in vitro* or *in vivo* transcription and translation of these sequences results in the production of atrD. Synthetic and semi-synthetic sequences encoding atrD may be constructed by techniques well known in the art. See Brown et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., 68:109-151. atrD-encoding DNA, or portions thereof, may be generated using a conventional DNA synthesizing apparatus such as the Applied Biosystems Model 380A, 380B, 394 or 3948 DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404).

Owing to the natural degeneracy of the genetic code, the skilled artisan will recognize that a sizable yet

definite number of nucleic acid sequences may be constructed which encode atrD. All such nucleic acid sequences are provided by the present invention. These sequences can be prepared by a variety of methods and, therefore, the invention is not limited to any particular preparation means. The nucleic acid sequences of the invention can be produced by a number of procedures, including DNA synthesis, cDNA cloning, genomic cloning, polymerase chain reaction (PCR) technology, or a combination of these approaches. These and other techniques are described by Maniatis, et al., *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York (1989), or *Current Protocols in Molecular Biology* (F. M. Ausubel et al., 1989 and supplements). The contents of both of these references are incorporated herein by reference.

In another aspect, this invention provides the cDNA encoding atrD, which may be obtained by synthesizing the desired portion of SEQ ID NO:1 or by following the procedure carried out by Applicants. This procedure involved construction of a cosmid genomic DNA library from *Aspergillus nidulans* strain OC-1, a mutant derived from A42355. This library was screened for genes related to MDRs using a homologous probe generated by PCR. Degenerate PCR primers directed towards amplification of DNA sequences encoding highly conserved regions found in the ATP-binding domain of several MDR genes were synthesized. PCR using these primers and *Aspergillus nidulans* genomic DNA as template produced an approximately 400 base pair DNA fragment. The DNA sequence of this fragment was highly homologous to the ATP-binding region of several MDRs as predicted. This fragment was used as a hybridization probe to identify cosmid clones containing the entire atrD gene. A subclone from one such cosmid containing the entire atrD gene was sequenced to ascertain the entire sequence of atrD.

To effect the translation of atrD-encoding mRNA, one inserts the natural, synthetic, or semi-synthetic atrD-encoding DNA sequence into any of a large number of appropriate expression vectors through the use of appropriate restriction endonucleases and DNA ligases. Synthetic and semi-synthetic atrD-encoding DNA sequences can be designed, and natural atrD-encoding nucleic acid can be modified, to possess restriction endonuclease cleavage sites to facilitate isolation from and integration into these vectors. Particular restriction endonucleases employed will be dictated by the restriction endonuclease cleavage pattern of the expression vector utilized. Restriction enzyme sites are chosen so as to properly orient the atrD-encoding DNA with the control sequences to achieve proper in-frame transcription and translation of the atrD molecule. The atrD-encoding DNA must be positioned so as to be in proper reading frame with the promoter and ribosome binding site of the expression vector, both of which are functional in the host cell in which atrD is to be expressed.

Expression of atrD in fungal cells, such as *Saccharomyces cerevisiae* is preferred. Suitable promoter sequences for use with yeast hosts include the promoters for 3-phosphoglycerate kinase (found on plasmid pAP12BD (ATCC 53231) and described in U.S. Patent No. 4,935,350, June 19, 1990) or other glycolytic enzymes such as enolase (found on plasmid pAC1 (ATCC 39532)), glyceraldehyde-3-phosphate dehydrogenase (derived from plasmid pHcGAPC1 (ATCC 57090, 57091)), hexokinase, pyruvate decarboxylase, phosphofructokinase, glucose-6-phosphate isomerase, 3-phosphoglycerate mutase, pyruvate kinase, triosephosphate isomerase, phosphoglucose isomerase, and glucokinase. Inducible yeast promoters have the additional advantage of transcription controlled by growth conditions. Such promoters include the promoter regions for alcohol dehydrogenase 2, isocytochrome C, acid phosphotase,

degradative enzymes associated with nitrogen metabolism, metallothionein (contained on plasmid vector pCL28XhoLHBPV (ATCC 39475), United States Patent No. 4,840,896), glycerinaldehyde 3-phosphate dehydrogenase, and enzymes
5 responsible for maltose and galactose utilization (GAL1 found on plasmid pRY121 (ATCC 37658) and on plasmid pPST5, described below). Suitable vectors and promoters for use in yeast expression are further described by R. Hitzeman et al., in European Patent Publication No. 73,657A. Yeast
10 enhancers such as the UAS Gal enhancer from *Saccharomyces cerevisiae* (found in conjunction with the CYC1 promoter on plasmid YEpsec--hI1beta, ATCC 67024), also are advantageously used with yeast promoters.

A variety of expression vectors useful in the present
15 invention are well known in the art. For expression in *Saccharomyces*, the plasmid YRp7, for example, (ATCC-40053, Stinchcomb et al., 1979, *Nature* 282:39; Kingsman et al., 1979, *Gene* 7:141 ; Tschemper et al., 1980, *Gene* 10:157) is commonly used. This plasmid contains the *trp* gene which
20 provides a selection marker for a mutant strain of yeast lacking the ability to grow in tryptophan, for example ATCC 44076 or PEP4-1 (Jones, 1977, *Genetics* 85:12).

Expression vectors useful in the expression of *atrD* can be constructed by a number of methods. For example, the
25 cDNA sequence encoding *atrD* can be synthesized using DNA synthesis techniques such as those described above. Such synthetic DNA can be synthesized to contain cohesive ends that allow facile cloning into an appropriately digested expression vector. For example, the cDNA encoding *atrD* can
30 be synthesized to contain *NotI* cohesive ends. Such a synthetic DNA fragment can be ligated into a *NotI*-digested expression vector such as pYES-2 (Invitrogen Corp., San Diego CA 92121).

An expression vector can also be constructed in the
35 following manner. Logarithmic phase *Aspergillus nidulans*

cells are disrupted by grinding under liquid nitrogen according to the procedure of Minuth et al., 1982 (*Current Genetics* 5:227-231). *Aspergillus nidulans* mRNA is preferably isolated from the disrupted cells using the QuickPrep[®] mRNA Purification Kit (Pharmacia Biotech) according to the instructions of the manufacturer. cDNA is produced from the isolated mRNA using the TimeSaver[®] cDNA Synthesis Kit (Pharmacia Biotech) using oligo (dT) according to the procedure described by the manufacturer. In this process an *EcoRI/NotI* adapter (Stratagene, Inc.) is ligated to each end of the double stranded cDNA. The adapter modified cDNA is ligated into the vector Lambda Zap^RII[®] using the Predigested Lambda Zap^RII[®]/*EcoRI*/CIAP Cloning Kit (Stratagene, Inc.) according to the instructions of the manufacturer to create a cDNA library.

The library is screened for full-length cDNA encoding atrD using a ³²P-radiolabeled fragment of the atrD gene. In this manner, a full-length cDNA clone is recovered from the *Aspergillus nidulans* cDNA library. A full-length cDNA clone recovered from the library is removed from the Lambda Zap^RII[®] vector by digestion with the restriction endonuclease *NotI* which produces a DNA fragment encoding atrD. The atrD encoding fragment is subcloned into plasmid pYES2 for expression studies. In this plasmid the atrD gene is operably linked to the *Saccharomyces cerevisiae* GAL1 promoter at the 5' end, and the yeast *cyc1* transcription terminator at the 3' end. This plasmid further comprises the ColE1 origin of replication (ColE1) which allows replication in *Escherichia coli* host cells, and the ampicillin resistance gene (Amp) for selection of *E. coli* cells transformed with the plasmid grown in the presence of ampicillin. The expression plasmid further comprises the yeast 2 μ origin of replication (2 μ ori) allowing replication in yeast host cells, the yeast URA3 gene for selection of *S. cerevisiae* cells transformed with the plasmid grown in a

medium lacking uracil, and the origin of replication from the f1 filamentous phage.

In a preferred embodiment of the invention *Saccharomyces cerevisiae* INVSc1 or INVSc2 cells (Invitrogen Corp., Sorrento Valley Blvd., San Diego CA 92121) are employed as host cells, but numerous other cell lines are available for this use. The transformed host cells are plated on an appropriate medium under selective pressure (minimal medium lacking uracil). The cultures are then incubated for a time and temperature appropriate to the host cell line employed.

The techniques involved in the transformation of yeast cells such as *Saccharomyces cerevisiae* cells are well known in the art and may be found in such general references as Ausubel et al., *Current Protocols in Molecular Biology* (1989), John Wiley & Sons, New York, NY and supplements. The precise conditions under which the transformed yeast cells are cultured is dependent upon the nature of the yeast host cell line and the vectors employed.

Nucleic acid, either RNA or DNA, which encodes atrD, or a portion thereof, is also useful in producing nucleic acid molecules useful in diagnostic assays for the detection of atrD mRNA, atrD cDNA, or atrD genomic DNA. Further, nucleic acid, either RNA or DNA, which does not encode atrD, but which nonetheless is capable of hybridizing with atrD-encoding DNA or RNA is also useful in such diagnostic assays. These nucleic acid molecules may be covalently labeled by known methods with a detectable moiety such as a fluorescent group, a radioactive atom or a chemiluminescent group. The labeled nucleic acid is then used in conventional hybridization assays, such as Southern or Northern hybridization assays, or polymerase chain reaction assays (PCR), to identify hybridizing DNA, cDNA, or RNA molecules. PCR assays may also be performed using unlabeled nucleic acid molecules. Such assays may be employed to

identify atrD vectors and transformants and in *in vitro* diagnosis to detect atrD-like mRNA, cDNA, or genomic DNA from other organisms.

United States Patent Application Serial. No. 08/111680,
5 the entire contents of which are hereby incorporated herein by reference, describes the use of combination therapy involving an antifungal agent possessing a proven spectrum of activity, with a fungal MDR inhibitor to treat fungal infections. This combination therapy approach enables an
10 extension of the spectrum of antifungal activity for a given antifungal compound which previously had only demonstrated limited clinically relevant antifungal activity. Similarly, compounds with demonstrated antifungal activity can also be potentiated by a fungal MDR inhibitor such that the
15 antifungal activity of these compounds is extended to previously resistant species. To identify compounds useful in such combination therapy the present invention provides an assay method for identifying compounds with *Aspergillus nidulans* MDR inhibition activity. Host cells that express
20 atrD provide an excellent means for the identification of compounds useful as inhibitors of *Aspergillus nidulans* MDR activity. Generally, the assay utilizes a culture of a yeast cell transformed with a vector which provides expression of atrD. The expression of atrD by the host cell
25 enables the host cell to grow in the presence of an antifungal compound to which the yeast cell is sensitive to in the untransformed state. Thus, the transformed yeast cell culture is grown in the presence of i) an antifungal agent to which the untransformed yeast cell is sensitive,
30 but to which the transformed host cell is resistant, and ii) a compound that is suspected of being an MDR inhibitor. The effect of the suspected MDR inhibitor is measured by testing for the ability of the antifungal compound to inhibit the growth of the transformed yeast cell. Such
35 inhibition will occur if the suspected *Aspergillus nidulans*

MDR inhibitor blocks the ability of atrD to prevent the antifungal compound from acting on the yeast cell. An illustrative example of such an assay is provided in Example 3.

5 In order to illustrate more fully the operation of this invention, the following examples are provided, but are not to be construed as a limitation on the scope of the invention.

10 Example 1

Source of the atrD-Encoding Genomic DNA and cDNA of
Aspergillus nidulans

15 Genomic DNA encoding atrD, or the corresponding cDNA sequence (presented in SEQ ID NO:1), may be from a natural sequence, a synthetic source or a combination of both ("semi-synthetic sequence"). The *in vitro* or *in vivo* transcription and translation of these sequences results in the production of atrD. Synthetic and semi-synthetic
20 sequences encoding atrD may be constructed by techniques well known in the art. See Brown et al. (1979) *Methods in Enzymology*, Academic Press, N.Y., 68:109-151. atrD-encoding DNA, or portions thereof, may be generated using a conventional DNA synthesizing apparatus such as the Applied
25 Biosystems Model 380A, 380B, 384 or 3848 DNA synthesizers (commercially available from Applied Biosystems, Inc., 850 Lincoln Center Drive, Foster City, CA 94404). The polymerase chain reaction is especially useful in generating these DNA sequences. PCR primers are constructed which
30 include the translational start (ATG) and translational stop codon (TAG) of atrD. Restriction enzyme sites may be included on these PCR primers outside of the atrD coding region to facilitate rapid cloning into expression vectors. *Aspergillus nidulans* genomic DNA is used as the PCR template
35 for synthesis of atrD including introns which is useful for

expression studies in closely related fungi. In contrast, cDNA is used as the PCR template for synthesis of atrD devoid of introns which is useful for expression in foreign hosts such as *Saccharomyces cerevisiae* or bacterial hosts
5 such as *Escherichia coli*.

Example 2

Expression of the atrD Protein

10 *Saccharomyces cerevisiae* INVSc1 cells (Invitrogen Corp., San Diego CA 92191) are transformed with the plasmid containing atrD by the technique described by J. D. Beggs, 1988, *Nature* 275:104-109). The transformed yeast cells are grown in a broth medium containing YNB/CSM-Ura/raf (YNB/CSM-
15 Ura [Yeast Nitrogen Base (Difco Laboratories, Detroit, MI) supplemented with CSM-URA (Bio 101, Inc.)] supplemented with 4% raffinose) at 28°C in a shaker incubator until the culture is saturated. To induce expression of atrD, a portion of the culture is used to inoculate a flask
20 containing YNB/CSM-Ura medium supplemented with 2% galactose (YNB/CSM-Ura/gal) rather than raffinose as the sole carbon source. The inoculated flask is incubated at 28°C for about 16 hours.

25

Example 3

Antifungal Potentiator Assay

Approximately 1×10^6 cells of a *Saccharomyces cerevisiae* INVSc1 culture expressing atrD are delivered to
30 each of several agar plates containing YNB/CSM-Ura/gal. The agar surface is allowed to dry in a biohazard hood.

An antifungal compound that the untransformed yeast cell is typically sensitive to is dissolved in an appropriate solvent at a concentration that is biologically
35 effective. Twenty μ l of the solution is delivered to an

antibiotic susceptibility test disc (Difco Laboratories, Detroit, MI). After addition of the antifungal solution the disc is allowed to air dry in a biohazard hood. When dry, the disc is placed on the surface of the petri plates
5 containing the transformed *Saccharomyces cerevisiae* INVSc1 cells.

Compounds to be tested for the ability to inhibit atrD are dissolved in dimethylsulfoxide (DMSO). The amount of compound added to the DMSO depends on the solubility of the
10 individual compound to be tested. Twenty μ l of the suspensions containing a compound to be tested are delivered to an antibiotic susceptibility test disc (Difco Laboratories, Detroit, MI). The disc is then placed on the surface of the dried petri plates containing the transformed
15 *Saccharomyces cerevisiae* INVSc1 cells approximately 2 cm from the antifungal-containing disc. Petri plates containing the two discs are incubated at 28°C for about 16-48 hours.

Following this incubation period, the petri plates are
20 examined for zones of growth inhibition around the discs. A zone of growth inhibition near the antifungal disc on the test plate indicates that the compound being tested for MDR inhibition activity blocks the activity of atrD and allows the antifungal compound to inhibit the growth of the yeast
25 host cell. Such compounds are said to possess MDR inhibition activity. Little or no zone of growth inhibition indicates that the test compound does not block MDR activity and, thus, atrD is allowed to act upon the antifungal compound to prevent its activity upon the host cell.

30

Example 4

Screen For Novel Antifungal Compounds

A plasmid molecule is constructed which contains DNA
35 sequence information required for replication and genetic

transformation in *E. coli* (e.g. ampicillin resistance). The plasmid also comprises DNA sequences encoding a marker for selection in fungal cells (e.g. hygromycin B phosphotransferase, phleomycin resistance, G418 resistance) under the control of an *A. nidulans* promoter. Additionally, the plasmid contains an internal portion of the *atrD* gene (e.g. about 3000 base pairs which lack 500 base pairs at the N-terminal end, and about 500 base pairs at the C-terminal end of the coding region specified by SEQ ID NO:1). The *atrD* gene fragment enables a single crossover gene disruption when transformed or otherwise introduced into *A. nidulans*.

Alternatively, a 5 kilobase pair to 6 kilobase pair region of *A. nidulans* genomic DNA containing the *atrD* gene is subcloned into the aforementioned plasmid. Then, a central portion of the *atrD* gene is removed and replaced with a selectable marker, such as hygromycin B phosphotransferase, for a double crossover gene replacement.

Gene disruption and gene replacement procedures for *A. nidulans* are well known in the art (See e.g. May et al, *J. Cell Biol.* 101, 712, 1985; Jones and Sealy-Lewis, *Curr. Genet.* 17, 81, 1990). Transformants are recovered on an appropriate selection medium, for example, hygromycin (if hygromycin B gene is used in the construction of disruption cassette). Gene replacement, or gene disruption, is verified by any suitable method, for example, by Southern blot hybridization.

Gene disruption or gene replacement strains are rendered hypersensitive to antifungal compounds, and are useful in screens for new antifungal compounds in whole cell growth inhibition studies.

- 1 -

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Skatrud, Paul L.
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Peery, Robert B.
Andrade, Alan C.

(ii) TITLE OF INVENTION: Multiple Drug Resistance Gene atrD of
Aspergillus Nidulans

(iii) NUMBER OF SEQUENCES: 3

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(F) ZIP: 46285

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
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(viii) ATTORNEY/AGENT INFORMATION:

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(C) REFERENCE/DOCKET NUMBER: X-11766

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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 4002 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

WO 99/32608

PCT/US98/27300

- 2 -

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

(ix) FEATURE:

(A) NAME/KEY: CDS

(B) LOCATION: 1..4002

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

ATG TCC CCG CTA GAG ACA AAT CCC CTT TCG CCA GAG ACT GCT ATG CGC	48
Met Ser Pro Leu Glu Thr Asn Pro Leu Ser Pro Glu Thr Ala Met Arg	
1 5 10 15	
GAA CCT GCT GAG ACT TCA ACG ACG GAG GAG CAA GCT TCT ACA CCA CAC	96
Glu Pro Ala Glu Thr Ser Thr Thr Glu Glu Gln Ala Ser Thr Pro His	
20 25 30	
GCT GCG GAC GAG AAG AAA ATC CTC AGC GAC CTC TCG GCT CCA TCT AGT	144
Ala Ala Asp Glu Lys Lys Ile Leu Ser Asp Leu Ser Ala Pro Ser Ser	
35 40 45	
ACT ACA GCA ACC CCC GCA GAC AAG GAG CAC CGT CCT AAA TCG TCG TCC	192
Thr Thr Ala Thr Pro Ala Asp Lys Glu His Arg Pro Lys Ser Ser Ser	
50 55 60	
AGC AAT AAT GCG GTC TCG GTC AAC GAA GTC GAT GCG CTT ATT GCG CAC	240
Ser Asn Asn Ala Val Ser Val Asn Glu Val Asp Ala Leu Ile Ala His	
65 70 75 80	
CTG CCA GAA GAC GAG AGG CAG GTC TTG AAG ACG CAG CTG GAG GAG ATC	288
Leu Pro Glu Asp Glu Arg Gln Val Leu Lys Thr Gln Leu Glu Glu Ile	
85 90 95	
AAA GTA AAC ATC TCC TTC TTC GGT CTC TGG CGG TAT GCA ACA AAG ATG	336
Lys Val Asn Ile Ser Phe Phe Gly Leu Trp Arg Tyr Ala Thr Lys Met	
100 105 110	
GAT ATA CTT ATC ATG GTA ATC AGT ACA ATC TGT GCC ATT GCT GCC GCG	384
Asp Ile Leu Ile Met Val Ile Ser Thr Ile Cys Ala Ile Ala Ala Ala	
115 120 125	
TCG ACT TTC CAG AGG ATA ATG TTA TAT CAA ATC TCG TAC GAC GAG TTC	432
Ser Thr Phe Gln Arg Ile Met Leu Tyr Gln Ile Ser Tyr Asp Glu Phe	
130 135 140	
TAT GAT GAA TTG ACC AAG AAC GTA CTG TAC TTC GTA TAC CTC GGT ATC	480
Tyr Asp Glu Leu Thr Lys Asn Val Leu Tyr Phe Val Tyr Leu Gly Ile	
145 150 155 160	

WO 99/32608

PCT/US98/27300

- 3 -

GGC GAG TTT GTC ACT GTC TAT GTT AGT ACT GTT GGC TTC ATC TAT ACC Gly Glu Phe Val Thr Val Tyr Val Ser Thr Val Gly Phe Ile Tyr Thr 165 170 175	528
GGA GAA CAC GCC ACG CAG AAG ATC CGC GAG TAT TAC CTT GAG TCT ATC Gly Glu His Ala Thr Gln Lys Ile Arg Glu Tyr Tyr Leu Glu Ser Ile 180 185 190	576
CTG CGC CAG AAC ATT GGC TAT TTT GAT AAA CTC GGT GCC GGG GAA GTG Leu Arg Gln Asn Ile Gly Tyr Phe Asp Lys Leu Gly Ala Gly Glu Val 195 200 205	624
ACC ACC CGT ATA ACA GCC GAT ACA AAC CTT ATC CAG GAT GGC ATT TCG Thr Thr Arg Ile Thr Ala Asp Thr Asn Leu Ile Gln Asp Gly Ile Ser 210 215 220	672
GAG AAG GTC GGT CTC ACT TTG ACT GCC CTG GCG ACA TTC GTG ACA GCA Glu Lys Val Gly Leu Thr Leu Thr Ala Leu Ala Thr Phe Val Thr Ala 225 230 235 240	720
TTC ATT ATC GCC TAC GTC AAA TAC TGG AAG TTG GCT CTA ATT TGC AGC Phe Ile Ile Ala Tyr Val Lys Tyr Trp Lys Leu Ala Leu Ile Cys Ser 245 250 255	768
TCA ACA ATT GTG GCC CTC GTT CTC ACC ATG GGC GGT GGT TCT CAG TTT Ser Thr Ile Val Ala Leu Val Leu Thr Met Gly Gly Gly Ser Gln Phe 260 265 270	816
ATC ATC AAG TAC AGC AAA AAG TCG CTT GAC AGC TAC GGT GCA GGC GGC Ile Ile Lys Tyr Ser Lys Lys Ser Leu Asp Ser Tyr Gly Ala Gly Gly 275 280 285	864
ACT GTT GCG GAA GAG GTC ATC AGC TCC ATC AGA AAT GCC ACA GCG TTT Thr Val Ala Glu Glu Val Ile Ser Ser Ile Arg Asn Ala Thr Ala Phe 290 295 300	912
GGC ACC CAA GAC AAG CTT GCG AAG CAG TAT GAG GTC CAC TTA GAC GAA Gly Thr Gln Asp Lys Leu Ala Lys Gln Tyr Glu Val His Leu Asp Glu 305 310 315 320	960
GCT GAG AAA TGG GGA ACA AAG AAC CAG ATT GTC ATG GGT TTC ATG ATT Ala Glu Lys Trp Gly Thr Lys Asn Gln Ile Val Met Gly Phe Met Ile 325 330 335	1008
GGC GCC ATG TTT GGC CTT ATG TAC TCG AAC TAC GGT CTT GGC TTC TGG Gly Ala Met Phe Gly Leu Met Tyr Ser Asn Tyr Gly Leu Gly Phe Trp 340 345 350	1056
ATG GGT TCT CGT TTC CTG GTA GAT GGT GCA GTC GAT GTG GGT GAT ATT Met Gly Ser Arg Phe Leu Val Asp Gly Ala Val Asp Val Gly Asp Ile 355 360 365	1104
CTC ACA GTT CTC ATG GCC ATC TTG ATC GGA TCG TTC TCC TTG GGG AAC	1152

WO 99/32608

PCT/US98/27300

- 4 -

Leu Thr Val Leu Met Ala Ile Leu Ile Gly Ser Phe Ser Leu Gly Asn	
370	375 380
GTT AGT CCA AAT GCT CAA GCA TTT ACA AAC GCT GTG GCC GCG GCC GCA	1200
Val Ser Pro Asn Ala Gln Ala Phe Thr Asn Ala Val Ala Ala Ala	
385	390 395 400
AAG ATA TTT GGA ACG ATC GAT CGC CAG TCC CCA TTA GAT CCA TAT TCG	1248
Lys Ile Phe Gly Thr Ile Asp Arg Gln Ser Pro Leu Asp Pro Tyr Ser	
405	410 415
AAC GAA GGG AAG ACG CTC GAC CAT TTT GAG GGC CAC ATT GAG TTA CGC	1296
Asn Glu Gly Lys Thr Leu Asp His Phe Glu Gly His Ile Glu Leu Arg	
420	425 430
AAT GTC AAG CAT ATT TAC CCA TCT AGA CCC GAG GTC ACC GTC ATG GAG	1344
Asn Val Lys His Ile Tyr Pro Ser Arg Pro Glu Val Thr Val Met Glu	
435	440 445
GAT GTT TCT CTG TCA ATG CCC GCT GGA AAA ACA ACC GCT TTA GTC GGC	1392
Asp Val Ser Leu Ser Met Pro Ala Gly Lys Thr Thr Ala Leu Val Gly	
450	455 460
CCC TCT GGC TCT GGA AAA AGT ACG GTG GTC GGC TTG GTT GAG CGA TTC	1440
Pro Ser Gly Ser Gly Lys Ser Thr Val Val Gly Leu Val Glu Arg Phe	
465	470 475 480
TAC ATG CCT GTT CGC GGT ACG GTT TTG CTG GAT GGC CAT GAC ATC AAG	1488
Tyr Met Pro Val Arg Gly Thr Val Leu Leu Asp Gly His Asp Ile Lys	
485	490 495
GAC CTC AAT CTC CGC TGG CTT CGC CAA CAG ATC TCT TTG GTT AGC CAG	1536
Asp Leu Asn Leu Arg Trp Leu Arg Gln Gln Ile Ser Leu Val Ser Gln	
500	505 510
GAG CCT GTT CTT TTT GGC ACG ACG ATT TAT AAG AAT ATT AGG CAC GGT	1584
Glu Pro Val Leu Phe Gly Thr Thr Ile Tyr Lys Asn Ile Arg His Gly	
515	520 525
CTC ATC GGC ACA AAG TAC GAG AAT GAA TCC GAG GAT AAG GTC CGG GAA	1632
Leu Ile Gly Thr Lys Tyr Glu Asn Glu Ser Glu Asp Lys Val Arg Glu	
530	535 540
CTC ATC GAG AAC GCG GCA AAA ATG GCG AAT GCT CAT GAC TTT ATT ACT	1680
Leu Ile Glu Asn Ala Ala Lys Met Ala Asn Ala His Asp Phe Ile Thr	
545	550 555 560
GCC TTG CCT GAA GGT TAT GAG ACC AAT GTT GGG CAG CGT GGC TTT CTC	1728
Ala Leu Pro Glu Gly Tyr Glu Thr Asn Val Gly Gln Arg Gly Phe Leu	
565	570 575
CTT TCA GGT GGC CAG AAA CAG CGC ATT GCA ATC GCC CGT GCC GTT GTT	1776
Leu Ser Gly Gly Gln Lys Gln Arg Ile Ala Ile Ala Arg Ala Val Val	

WO 99/32608

PCT/US98/27300

- 5 -

				580					585					590					
AGT	GAC	CCA	AAA	ATC	CTG	CTC	CTG	GAT	GAA	GCT	ACT	TCG	GCC	TTG	GAC		1824		
Ser	Asp	Pro	Lys	Ile	Leu	Leu	Leu	Asp	Glu	Ala	Thr	Ser	Ala	Leu	Asp				
				595					600					605					
ACA	AAA	TCC	GAA	GGC	GTG	GTT	CAA	GCA	GCT	TTG	GAG	AGG	GCA	GCT	GAA		1872		
Thr	Lys	Ser	Glu	Gly	Val	Val	Gln	Ala	Ala	Leu	Glu	Arg	Ala	Ala	Glu				
				610					615					620					
GGC	CGA	ACT	ACT	ATT	GTG	ATC	GCT	CAT	CGC	CTT	TCC	ACG	ATC	AAA	ACG		1920		
Gly	Arg	Thr	Thr	Ile	Val	Ile	Ala	His	Arg	Leu	Ser	Thr	Ile	Lys	Thr				
				625					630					635					640
GCG	CAC	AAC	ATT	GTG	GTT	CTG	GTC	AAT	GGC	AAA	ATT	GCT	GAA	CAA	GGA		1968		
Ala	His	Asn	Ile	Val	Val	Leu	Val	Asn	Gly	Lys	Ile	Ala	Glu	Gln	Gly				
				645					650					655					
ACT	CAC	GAT	GAA	TTG	GTT	GAC	CGC	GGA	GGC	GCT	TAT	CGC	AAA	CTT	GTG		2016		
Thr	His	Asp	Glu	Leu	Val	Asp	Arg	Gly	Gly	Ala	Tyr	Arg	Lys	Leu	Val				
				660					665					670					
GAG	GCT	CAA	CGT	ATC	AAT	GAA	CAG	AAG	GAA	GCT	GAC	GCC	TTG	GAG	GAC		2064		
Glu	Ala	Gln	Arg	Ile	Asn	Glu	Gln	Lys	Glu	Ala	Asp	Ala	Leu	Glu	Asp				
				675					680					685					
GCC	GAC	GCT	GAG	GAT	CTC	ACG	AAT	GCA	GAT	ATT	GCC	AAA	ATC	AAA	ACT		2112		
Ala	Asp	Ala	Glu	Asp	Leu	Thr	Asn	Ala	Asp	Ile	Ala	Lys	Ile	Lys	Thr				
				690					695					700					
GCG	TCA	AGC	GCA	TCA	TCC	GAT	CTC	GAC	GGA	AAA	CCC	ACA	ACC	ATT	GAC		2160		
Ala	Ser	Ser	Ala	Ser	Ser	Asp	Leu	Asp	Gly	Lys	Pro	Thr	Thr	Ile	Asp				
				705					710					715					720
CGC	ACG	GGC	ACC	CAC	AAG	TCT	GTT	TCC	AGC	GCG	ATT	CTT	TCT	AAA	AGA		2208		
Arg	Thr	Gly	Thr	His	Lys	Ser	Val	Ser	Ser	Ala	Ile	Leu	Ser	Lys	Arg				
				725					730					735					
CCC	CCC	GAA	ACA	ACT	CCG	AAA	TAC	TCA	TTA	TGG	ACG	CTG	CTC	AAA	TTT		2256		
Pro	Pro	Glu	Thr	Thr	Pro	Lys	Tyr	Ser	Leu	Trp	Thr	Leu	Leu	Lys	Phe				
				740					745					750					
GTT	GCT	TCC	TTC	AAC	CGC	CCT	GAA	ATC	CCG	TAC	ATG	CTC	ATC	GGT	CTT		2304		
Val	Ala	Ser	Phe	Asn	Arg	Pro	Glu	Ile	Pro	Tyr	Met	Leu	Ile	Gly	Leu				
				755					760					765					
GTC	TTC	TCA	GTG	TTA	GCT	GGT	GGT	GGC	CAA	CCC	ACG	CAA	GCA	GTG	CTA		2352		
Val	Phe	Ser	Val	Leu	Ala	Gly	Gly	Gly	Gln	Pro	Thr	Gln	Ala	Val	Leu				
				770					775					780					
TAT	GCT	AAA	GCC	ATC	AGC	ACA	CTC	TCG	CTC	CCA	GAA	TCA	CAA	TAT	AGC		2400		
Tyr	Ala	Lys	Ala	Ile	Ser	Thr	Leu	Ser	Leu	Pro	Glu	Ser	Gln	Tyr	Ser				
				785					790					795					800

- 6 -

AAG CTT CGA CAT GAT GCG GAT TTC TGG TCA TTG ATG TTC TTC GTG GTT	2448
Lys Leu Arg His Asp Ala Asp Phe Trp Ser Leu Met Phe Phe Val Val	
805 810 815	
GGT ATC ATT CAG TTT ATC ACG CAG TCA ACC AAT GGT GCT GCA TTT GCC	2496
Gly Ile Ile Gln Phe Ile Thr Gln Ser Thr Asn Gly Ala Ala Phe Ala	
820 825 830	
GTA TGC TCC GAG AGA CTT ATT CGT CGC GCG AGA AGC ACT GCC TTT CGG	2544
Val Cys Ser Glu Arg Leu Ile Arg Arg Ala Arg Ser Thr Ala Phe Arg	
835 840 845	
ACG ATA CTC CGT CAA GAC ATT GCT TTC TTT GAC AAG GAA GAG AAT AGC	2592
Thr Ile Leu Arg Gln Asp Ile Ala Phe Phe Asp Lys Glu Glu Asn Ser	
850 855 860	
ACC GGC GCT CTG ACC TCT TTC CTG TCC ACC GAG ACG AAG CAT CTC TCC	2640
Thr Gly Ala Leu Thr Ser Phe Leu Ser Thr Glu Thr Lys His Leu Ser	
865 870 875 880	
GGT GTT AGC GGT GTG ACT CTA GGC ACG ATC TTG ATG ACC TCC ACG ACC	2688
Gly Val Ser Gly Val Thr Leu Gly Thr Ile Leu Met Thr Ser Thr Thr	
885 890 895	
CTA GGA GCG GCT ATC ATT ATT GCC CTG GCG ATT GGG TGG AAA TTG GCC	2736
Leu Gly Ala Ala Ile Ile Ala Leu Ala Ile Gly Trp Lys Leu Ala	
900 905 910	
TTA GTT TGT ATC TCG GTT GTG CCG GTT CTC CTG GCA TGC GGT TTC TAC	2784
Leu Val Cys Ile Ser Val Val Pro Val Leu Leu Ala Cys Gly Phe Tyr	
915 920 925	
CGA TTC TAT ATG CTA GCC CAG TTT CAA TCA CGC TCC AAG CTT GCT TAT	2832
Arg Phe Tyr Met Leu Ala Gln Phe Gln Ser Arg Ser Lys Leu Ala Tyr	
930 935 940	
GAG GGA TCT GCA AAC TTT GCT TGC GAG GCT ACA TCG TCT ATC CGC ACA	2880
Glu Gly Ser Ala Asn Phe Ala Cys Glu Ala Thr Ser Ser Ile Arg Thr	
945 950 955 960	
GTT GCG TCA TTA ACC CGG GAA AGG GAT GTC TGG GAG ATT TAC CAT GCC	2928
Val Ala Ser Leu Thr Arg Glu Arg Asp Val Trp Glu Ile Tyr His Ala	
965 970 975	
CAG CTT GAC GCA CAA GGC AGG ACC AGT CTA ATC TCT GTC TTG AGG TCA	2976
Gln Leu Asp Ala Gln Gly Arg Thr Ser Leu Ile Ser Val Leu Arg Ser	
980 985 990	
TCC CTG TTA TAT GCG TCG TCG CAG GCA CTT GTT TTC TTC TGC GTT GCG	3024
Ser Leu Leu Tyr Ala Ser Ser Gln Ala Leu Val Phe Phe Cys Val Ala	
995 1000 1005	

- 7 -

CTC GGG TTT TGG TAC GGA GGG ACA CTT CTT GGT CAC CAC GAG TAT GAC Leu Gly Phe Trp Tyr Gly Gly Thr Leu Leu Gly His His Glu Tyr Asp 1010 1015 1020	3072
ATT TTC CGC TTC TTT GTT TGT TTC TCC GAG ATT CTC TTT GGT GCT CAA Ile Phe Arg Phe Phe Val Cys Phe Ser Glu Ile Leu Phe Gly Ala Gln 1025 1030 1035 1040	3120
TCC GCG GGC ACC GTC TTT TCC TTT GCA CCA GAC ATG GGC AAG GCG AAG Ser Ala Gly Thr Val Phe Ser Phe Ala Pro Asp Met Gly Lys Ala Lys 1045 1050 1055	3168
AAT GCG GCC GCC GAA TTC CGA CGA CTG TTC GAC CGA AAG CCA CAA ATT Asn Ala Ala Ala Glu Phe Arg Arg Leu Phe Asp Arg Lys Pro Gln Ile 1060 1065 1070	3216
GAT AAC TGG TCT GAA GAG GGC GAG AAG CTC GAA ACG GTG GAA GGT GAA Asp Asn Trp Ser Glu Glu Gly Glu Lys Leu Glu Thr Val Glu Gly Glu 1075 1080 1085	3264
ATC GAA TTT AGG AAC GTG CAC TTC AGA TAC CCG ACC CGC CCA GAA CAG Ile Glu Phe Arg Asn Val His Phe Arg Tyr Pro Thr Arg Pro Glu Gln 1090 1095 1100	3312
CCT GTC CTG CGC GGC TTG GAC CTG ACC GTG AAG CCT GGA CAA TAT GTT Pro Val Leu Arg Gly Leu Asp Leu Thr Val Lys Pro Gly Gln Tyr Val 1105 1110 1115 1120	3360
GCG CTT GTC GGA CCC AGC GGT TGT GGC AAG AGT ACC ACC ATT GCA TTG Ala Leu Val Gly Pro Ser Gly Cys Gly Lys Ser Thr Thr Ile Ala Leu 1125 1130 1135	3408
CTT GAG CGC TTT TAC GAT GCG ATT GCC GGG TCC ATC CTT GTT GAT GGG Leu Glu Arg Phe Tyr Asp Ala Ile Ala Gly Ser Ile Leu Val Asp Gly 1140 1145 1150	3456
AAG GAC ATA AGT AAA CTA AAT ATC AAC TCC TAC CGC AGC TTT CTG TCA Lys Asp Ile Ser Lys Leu Asn Ile Asn Ser Tyr Arg Ser Phe Leu Ser 1155 1160 1165	3504
CTG GTC AGC CAG GAG CCG ACA CTG TAC CAG GGC ACC ATC AAG GAA AAC Leu Val Ser Gln Glu Pro Thr Leu Tyr Gln Gly Thr Ile Lys Glu Asn 1170 1175 1180	3552
ATC TTA CTT GGT ATT GTC GAA GAT GAC GTA CCG GAA GAA TTC TTG ATT Ile Leu Leu Gly Ile Val Glu Asp Asp Val Pro Glu Glu Phe Leu Ile 1185 1190 1195 1200	3600
AAG GCT TGC AAG GAC GCT AAT ATC TAC GAC TTC ATC ATG TCG CTC CCG Lys Ala Cys Lys Asp Ala Asn Ile Tyr Asp Phe Ile Met Ser Leu Pro 1205 1210 1215	3648
GAG GGC TTT AAT ACA GTT GTT GGC AGC AAG GGA GGC ATG TTG TCT GGC	3696

WO 99/32608

PCT/US98/27300

- 9 -

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

WO 99/32608

PCT/US98/27300

- 11 -

Gly Arg Thr Thr Ile Val Ile Ala His Arg Leu Ser Thr Ile Lys Thr
 625 630 635 640
 Ala His Asn Ile Val Val Leu Val Asn Gly Lys Ile Ala Glu Gln Gly
 645 650 655
 Thr His Asp Glu Leu Val Asp Arg Gly Gly Ala Tyr Arg Lys Leu Val
 660 665 670
 Glu Ala Gln Arg Ile Asn Glu Gln Lys Glu Ala Asp Ala Leu Glu Asp
 675 680 685
 Ala Asp Ala Glu Asp Leu Thr Asn Ala Asp Ile Ala Lys Ile Lys Thr
 690 695 700
 Ala Ser Ser Ala Ser Ser Asp Leu Asp Gly Lys Pro Thr Thr Ile Asp
 705 710 715 720
 Arg Thr Gly Thr His Lys Ser Val Ser Ser Ala Ile Leu Ser Lys Arg
 725 730 735
 Pro Pro Glu Thr Thr Pro Lys Tyr Ser Leu Trp Thr Leu Leu Lys Phe
 740 745 750
 Val Ala Ser Phe Asn Arg Pro Glu Ile Pro Tyr Met Leu Ile Gly Leu
 755 760 765
 Val Phe Ser Val Leu Ala Gly Gly Gly Gln Pro Thr Gln Ala Val Leu
 770 775 780
 Tyr Ala Lys Ala Ile Ser Thr Leu Ser Leu Pro Glu Ser Gln Tyr Ser
 785 790 795 800
 Lys Leu Arg His Asp Ala Asp Phe Trp Ser Leu Met Phe Phe Val Val
 805 810 815
 Gly Ile Ile Gln Phe Ile Thr Gln Ser Thr Asn Gly Ala Ala Phe Ala
 820 825 830
 Val Cys Ser Glu Arg Leu Ile Arg Arg Ala Arg Ser Thr Ala Phe Arg
 835 840 845
 Thr Ile Leu Arg Gln Asp Ile Ala Phe Phe Asp Lys Glu Glu Asn Ser
 850 855 860
 Thr Gly Ala Leu Thr Ser Phe Leu Ser Thr Glu Thr Lys His Leu Ser
 865 870 875 880
 Gly Val Ser Gly Val Thr Leu Gly Thr Ile Leu Met Thr Ser Thr Thr
 885 890 895
 Leu Gly Ala Ala Ile Ile Ile Ala Leu Ala Ile Gly Trp Lys Leu Ala

WO 99/32608

PCT/US98/27300

- 12 -

900	905	910
Leu Val Cys Ile Ser Val Val Pro Val Leu Leu Ala Cys Gly Phe Tyr 915 920 925		
Arg Phe Tyr Met Leu Ala Gln Phe Gln Ser Arg Ser Lys Leu Ala Tyr 930 935 940		
Glu Gly Ser Ala Asn Phe Ala Cys Glu Ala Thr Ser Ser Ile Arg Thr 945 950 955 960		
Val Ala Ser Leu Thr Arg Glu Arg Asp Val Trp Glu Ile Tyr His Ala 965 970 975		
Gln Leu Asp Ala Gln Gly Arg Thr Ser Leu Ile Ser Val Leu Arg Ser 980 985 990		
Ser Leu Leu Tyr Ala Ser Ser Gln Ala Leu Val Phe Phe Cys Val Ala 995 1000 1005		
Leu Gly Phe Trp Tyr Gly Gly Thr Leu Leu Gly His His Glu Tyr Asp 1010 1015 1020		
Ile Phe Arg Phe Phe Val Cys Phe Ser Glu Ile Leu Phe Gly Ala Gln 1025 1030 1035 1040		
Ser Ala Gly Thr Val Phe Ser Phe Ala Pro Asp Met Gly Lys Ala Lys 1045 1050 1055		
Asn Ala Ala Ala Glu Phe Arg Arg Leu Phe Asp Arg Lys Pro Gln Ile 1060 1065 1070		
Asp Asn Trp Ser Glu Glu Gly Glu Lys Leu Glu Thr Val Glu Gly Glu 1075 1080 1085		
Ile Glu Phe Arg Asn Val His Phe Arg Tyr Pro Thr Arg Pro Glu Gln 1090 1095 1100		
Pro Val Leu Arg Gly Leu Asp Leu Thr Val Lys Pro Gly Gln Tyr Val 1105 1110 1115 1120		
Ala Leu Val Gly Pro Ser Gly Cys Gly Lys Ser Thr Thr Ile Ala Leu 1125 1130 1135		
Leu Glu Arg Phe Tyr Asp Ala Ile Ala Gly Ser Ile Leu Val Asp Gly 1140 1145 1150		
Lys Asp Ile Ser Lys Leu Asn Ile Asn Ser Tyr Arg Ser Phe Leu Ser 1155 1160 1165		
Leu Val Ser Gln Glu Pro Thr Leu Tyr Gln Gly Thr Ile Lys Glu Asn 1170 1175 1180		

WO 99/32608

PCT/US98/27300

- 13 -

Ile Leu Leu Gly Ile Val Glu Asp Asp Val Pro Glu Glu Phe Leu Ile
 1185 1190 1195 1200

Lys Ala Cys Lys Asp Ala Asn Ile Tyr Asp Phe Ile Met Ser Leu Pro
 1205 1210 1215

Glu Gly Phe Asn Thr Val Val Gly Ser Lys Gly Gly Met Leu Ser Gly
 1220 1225 1230

Gly Gln Lys Gln Arg Val Ala Ile Ala Arg Ala Leu Leu Arg Asp Pro
 1235 1240 1245

Lys Ile Leu Leu Leu Asp Glu Ala Thr Ser Ala Leu Asp Ser Glu Ser
 1250 1255 1260

Glu Lys Val Val Gln Ala Ala Leu Asp Ala Ala Ala Arg Gly Arg Thr
 1265 1270 1275 1280

Thr Ile Ala Val Ala His Arg Leu Ser Thr Ile Gln Lys Ala Asp Val
 1285 1290 1295

Ile Tyr Val Phe Asp Gln Gly Lys Ile Val Glu Ser Gly Thr His Ser
 1300 1305 1310

Glu Leu Val Gln Lys Lys Gly Arg Tyr Tyr Glu Leu Val Asn Leu Gln
 1315 1320 1325

Ser Leu Gly Lys Gly His
 1330

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 4002 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: mRNA

(iii) HYPOTHETICAL: NO

(iv) ANTI-SENSE: NO

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

AUGUCCCCGC UAGAGACAAA UCCCCUUCG CCAGAGACUG CUAUGCGCGA ACCUGCUGAG 60

ACUUCAACGA CGGAGGAGCA AGCUUCUACA CCACACGCUG CGGACGAGAA GAAAAUCCUC 120

WO 99/32608

PCT/US98/27300

- 14 -

AGCGACCUCU CGGCUCCAUC UAGUACUACA GCAACCCCGG CAGACAAGGA GCACCGUCCU	180
AAAUCGUCGU CCAGCAAUAA UGCGGUCUCG GUCAACGAAG UCGAUGCGCU UAUUGCGCAC	240
CUGCCAGAAG ACGAGAGGCA GGUCUUGAAG ACGCAGCUGG AGGAGAUCAA AGUAAACAUC	300
UCCUUCUUCG GUCUCUGGCG GUAUGCAACA AAGAUGGAUA UACUUAUCAU GGUAUCAGU	360
ACAAUCUGUG CCAUUGCUGC CGCGUCGACU UCCAGAGGA UAAUGUUUAU UCAAUCUCG	420
UACGACGAGU UCUAUGAUGA AUUGACCAAG AACGUACUGU ACUUCGUUAU CCUCGGUAUC	480
GGCGAGUUUG UCACUGUCUA UGUUAGUACU GUUGGCUUCA UCUAUACCGG AGAACACGCC	540
ACGCAGAAGA UCCGCGAGUA UUACCUUGAG UCUAUCCUGC GCCAGAACAU UGGCUAUUUU	600
GAUAAACUCG GUGCCGGGGA AGUGACCACC CGUAUAACAG CCGAUACAAA CCUUAUCCAG	660
GAUGGCAUUU CGGAGAAGGU CGGUCUCACU UUGACUGCCC UGGCGACAUU CGUGACAGCA	720
UUCAUUAUCG CCUACGUCAA AUACUGGAAG UUGGCUCUAA UUUGCAGCUC AACAAUUGUG	780
GCCUCGUUC UCACCAUGGG CGGUGGUUCU CAGUUUAUCA UCAAGUACAG CAAAAGUCG	840
CUUGACAGCU ACGGUGCAGG CGGCACUGUU GCGGAAGAGG UCAUCAGCUC CAUCAGAAU	900
GCCACAGCGU UUGGCACCCA AGACAAGCUU GCGAAGCAGU AUGAGGUCCA CUUAGACGAA	960
GCUGAGAAAU GGGGAACAAA GAACCAGAUU GUCAUGGGUU UCAUGAUJGG CGCCAUGUUU	1020
GGCCUUAUGU ACUCGAACUA CGGUCUUGGC UUCUGGAUGG GUUCUCGUUU CCUGGUAGAU	1080
GGUGCAGUCG AUGUGGGUGA UAUUCUCACA GUUCUCAUGG CCAUCUUGAU CGGAUCGUUC	1140
UCCUUGGGGA ACGUUAGUCC AAAUGCUCAA GCAUUUACAA ACGCUGUGGC CGCGGCCGCA	1200
AAGAUUUUG GAACGAUCGA UCGCCAGUCC CCAUUAGAUC CAUAUUCGAA CGAAGGGAAG	1260
ACGCUCGACC AUUUUGAGGG CCACAUUGAG UUACGCAAUG UCAAGCAUUA UUACCCAUCU	1320
AGACCCGAGG UCACCGUCAU GGAGGAUGUU UCUCUGUCAU UGCCCCUGG AAAACAACC	1380
GCUUUAGUCG GCCCCUCUGG CUCUGGAAAA AGUACGGUGG UCGGCUUGGU UGAGCGAUUC	1440
UACAUGCCUG UUCGCGGUAC GGUUUUGCUG GAUGGCCAUG ACAUCAAGGA CCUCAUCUC	1500
CGCUGGCUUC GCCAACAGAU CUCUUUGGUU AGCCAGGAGC CUGUUCUUUU UGGCAGCAGG	1560
AUUUAUAAGA AUUUUAGGCA CGGUCUCAUC GGCACAAAGU ACGAGAAUGA AUCCGAGGAU	1620
AAGGUCCGGG AACUCAUCGA GAACGCGGCA AAAAUGGCGA AUGCUCAUGA CUUUAUUACU	1680
GCCUUGCCUG AAGGUUAUGA GACCAAUGUU GGGCAGCGUG GCUUUCUCCU UUCAGGUGGC	1740

WO 99/32608

PCT/US98/27300

- 15 -

CAGAAACAGC GCAUUGCAAU CGCCCGUGCC GUUGUUAGUG ACCCAAAAU CCUGCUCCUG	1800
GAUGAAGCUA CUUCGGCCUU GGACACAAA UCCGAAGGCG UGGUUCAAGC AGCUUUGGAG	1860
AGGGCAGCUG AAGGCCGAAC UACUAUUGUG AUCGCUCAUC GCCUUUCCAC GAUCAAAACG	1920
GCGCACAACA UUGUGGUUCU GGUCA AUGGC AAAAUJGUG AACAAGGAAC UCACGAUGAA	1980
UUGGUUGACC GCGGAGGCGC UUAUCGCAA CUUGUGGAGG CUCAACGU AU CAAUGAACAG	2040
AAGGAAGCUG ACGCCUUGGA GGACGCCGAC GCUGAGGAUC UCACGAAUGC AGAUUUGCC	2100
AAAUCAAAA CUGCGUCAAG CGCAUCAUCC GAUCUCGACG GAAAACCCAC AACCAUUGAC	2160
CGCACGGGCA CCCACAAGUC UGUUCCAGC GCGAUUCUUU CUAAAAGACC CCCCAGAAACA	2220
ACUCCGAAAU ACUCAUUAUG GACGCUGCUC AAUUGUUG CUUCCUCAA CCGCCUGAA	2280
AUCCCGUACA UGCUCAUCGG UCUUGUCUUC UCAGUGUAG CUGGUGGUGG CCAACCCAGC	2340
CAAGCAGUGC UAU AUGCUAA AGCCAUCAGC ACACUCUCGC UCCCAGAAUC ACAUAUAGC	2400
AAGCUUCGAC AUGAUGCGGA UUUCUGGUCA UUGAUGUUCU UCGUGGUUGG UAUCAUUCAG	2460
UUUAUCACGC AGUCAACCAA UGGUGCUGCA UUUGCCGU AU GCUCCGAGAG ACUUAUUCGU	2520
CGCGCGAGAA GCACUGCCUU UCGGACGAUA CUCCGUCAAG ACAUUGCUUU CUUUGACAAG	2580
GAAGAGAAUA GCACCGGCGC UCUGACCUCU UCCUGUCCA CCGAGACGAA GCAUCUCUCC	2640
GGUGUUAGCG GUGUGACUCU AGGCACGAUC UUGAUGACCU CCACGACCCU AGGAGCGGCU	2700
AUCAUUAUUG CCCUGGCGAU UGGUGGAAA UUGGCCUAG UUUGUAUCUC GGUUGUGCCG	2760
GUUCUCCUGG CAUGC GGUU CUACCGAUUC UAU AUGCUAG CCCAGUUUA AUCACGCUCC	2820
AAGCUUGCUU AUGAGGGAUC UGCAAACUUU GCUUGCGAGG CUACAUCGUC UAUCCGCACA	2880
GUUGCGUCAU UAACCCGGGA AAGGGAUGUC UGGGAGAUUU ACCAUGCCCA GCUUGACGCA	2940
CAAGGCAGGA CCAGUCUAAU CUCUGUCUUG AGGUCAUCCC UGUUAUAUGC GUCGUCGCAG	3000
GCACUUGUUU UCUUCUGCGU UGCGCUCGGG UUUUGGUACG GAGGGACACU UCUUGGUCAC	3060
CACGAGUAUG ACAUUUCCG CUUCUUUGUU UGUUUCUCCG AGAUUCUCUU UGGUGCUGAA	3120
UCCGCGGGCA CCGUCUUUUC CUUUGCACCA GACAUGGGCA AGGCGAAGAA UGCGGCCGCC	3180
GAAUCCGAC GACUGUUCGA CCGAAAGCCA CAAUUGAUA ACUGGUCUGA AGAGGGCGAG	3240
AAGCUCGAAA CGGUGGAAGG UGAAAUCGAA UUUAGGAACG UGCACUUCAG AUACCCGACC	3300

WO 99/32608

PCT/US98/27300

- 16 -

CGCCCAGAAC AGCCUGUCCU GCGCGGCUUG GACCUGACCG UGAAGCCUGG ACAAUAUGUU	3360
GCGCUUGUCG GACCCAGCGG UUGUGGCAAG AGUACCACCA UUGCAUUGCU UGAGCGCUUU	3420
UACGAUGCGA UUGCCGGGUC CAUCCUUGUU GAUGGGAAGG ACAUAAGUAA ACUAAAUAUC	3480
AACUCCUACC GCAGCUUUCU GUCACUGGUC AGCCAGGAGC CGACACUGUA CCAGGGCACC	3540
AUCAAGGAAA ACAUCUUACU UGGUAUUGUC GAAGAUGACG UACCGGAAGA AUUCUUGAUU	3600
AAGGCUUGCA AGGACGCUAA UAUCUACGAC UUCAUCAUGU CGCUCCCGGA GGGCUUUAU	3660
ACAGUUGUUG GCAGCAAGGG AGGCAUGUUG UCUGGCGGCC AAAAGCAACG UGUGGCCAUU	3720
GCCCGAGCCC UUCUUCGGGA UCCCAAAAUC CUUCUUCUCG AUGAAGCGAC GUCAGCCCUC	3780
GACUCCGAGU CAGAAAAGGU CGUCCAGGCG GCUUUGGAUG CCGCUGCCCG AGGCCGAACC	3840
ACAAUCGCCG UUGCACACCG ACUCAGCACG AUUCAAAAGG CGGACGUUUAU CUAUGUUUUC	3900
GACCAAGGCA AGAUCGUCGA AAGCGGAACG CACAGCGAAC UGGUCCAGAA AAAGGGCCGG	3960
UACUACGAGC UGGUCAACUU GCAGAGCUUG GGCAAGGGCC AU	4002

CLAIMS

We claim:

- 5 1. A DNA compound that comprises an isolated DNA sequence encoding SEQ ID NO: 2.
2. The DNA compound of Claim 1 which comprises the isolated DNA sequence which is SEQ ID NO: 1.
- 10 3. A vector comprising an isolated DNA sequence of Claim 1.
4. A vector comprising an isolated DNA sequence of Claim 2.
- 15 5. A method for constructing a transformed host cell capable of expressing SEQ ID NO: 2, said method comprising transforming a host cell with a recombinant DNA vector that comprises an isolated DNA sequence of Claim 1.
- 20 6. A method for expressing SEQ ID NO: 2 in a transformed host cell said method comprising culturing said transformed host cell of Claim 5 under conditions suitable for gene expression.
- 25 7. An isolated DNA molecule of Claim 1 or a portion thereof, which is labeled with a detectable moiety.
8. A host cell containing the vector of Claim 3.
- 30 9. A host cell containing the vector of Claim 4.
10. A method for determining the fungal MDR inhibition activity of a compound which comprises:
- 35 a) placing a culture of fungal cells, transformed with a vector capable of expressing atrD, in the presence of:

(i) an antifungal agent to which said fungal cell is resistant, but to which said fungal cell is sensitive in its untransformed state;

(ii) a compound suspected of possessing
5 *Aspergillus nidulans* MDR inhibition activity; and

b) determining the fungal MDR inhibition activity of said compound by measuring the ability of the antifungal agent to inhibit the growth of said fungal cell.

10 11. A method of Claim 10 wherein the fungal cell is *Saccharomyces cerevisiae*.

12. The protein of SEQ ID No. 2 in purified form.

15 13. A strain of *A. nidulans* wherein said strain carries a gene disruption or gene replacement at the *atrD* locus such that said strain does not produce the *atrD* protein product.

20 14. A method for identifying an antifungal compound comprising the steps of:

- a. culturing in the presence of a test compound a strain of claim 13;
- b. culturing said strain in the absence of said test compound; and
- 25 c. comparing the growth of said strain in step (a) with the growth in step (b).