ABC transporters and multidrug resistance in *Aspergillus nidulans*

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To Ivete, Diego e minha Mãe,
my eternal supporters

Stellingen

1. ABC transporters from *Aspergillus nidulans* can protect fungal cells against natural toxic compounds and xenobiotics. *This thesis.*
2. Cells possessing multidrug-efflux proteins as a protection mechanism against unrelated natural toxins have a competitive advantage in the chemical war between microorganisms in nature.
3. Understanding the physiological function(s) of ABC transporters facilitates to design strategies to overcome multidrug resistance in practice. *This thesis.*
4. ABC transporters from filamentous fungi can play a role in secretion of endogenous antibiotics. *This thesis.*
5. Different ABC transporters from *Aspergillus nidulans* can transport the same compound but can also have a distinct affinity for a specific substrate. *This thesis.*
6. *Aspergillus nidulans* is a suitable model to uncover substrate specificity of ABC proteins from other filamentous fungi, such as the human pathogens *Aspergillus fumigatus* and *Aspergillus flavus*. *This thesis.*
7. The *imaB* mutant of *Aspergillus nidulans* carries a mutation in a regulatory protein. *This thesis.*
8. The exciting part of being a molecular biologist is to re-build the "machine of life" without a manual of instruction.

Stellingen behorend bij het proefschrift van Alan C. Andrade: "ABC transporters and multidrug resistance in *Aspergillus nidulans*".
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Outline of this thesis

The ATP-binding cassette (ABC) transporters comprise a large and multifunctional family of proteins. ABC transporters are present from archae-bacteria to man but became especially known for their involvement in multidrug resistance (MDR) in tumour cells. MDR is often accompanied by a massive overproduction of ABC transporters. ABC transporters are also involved in various human diseases such as cystic fibrosis, adrenoleukodystrophy, the Zellweger syndrome, the Tangier disease and familial high-density lipoprotein deficiency. Furthermore, they play a role as peptide transporters in antigen presentation. The majority of the ABC transporters in higher organisms consists of two transmembrane domains (TMD), each with six predicted membrane spanning regions, and two nucleotide binding folds (NBF) in a two times two-domain configuration. The nucleotide binding domain can be either located at the amino terminus or at the carboxy terminus of the polypeptide, yielding proteins with a $[TMD\text{-}NBF]_2$ or $[NBF\text{-}TMD]_2$ configuration. In Chapter 1 an overview of the remarkable variety of cellular functions that these proteins can perform in all living cells, is presented.

The main goal of the studies presented in this thesis was to understand the role of ABC-transporter proteins in MDR of the filamentous fungus *Aspergillus nidulans*. Genetic and biochemical data previously generated in the Laboratory of Phytopathology, demonstrate that resistance to azole fungicides in laboratory-generated *ima* (imazalil-resistant) mutants is based on an increased energy-dependent efflux mechanism that prevents intracellular accumulation of the fungicide. This observation, provided the basis for the present work. Similar efflux mechanisms are described for human cancer cells with a MDR phenotype. MDR in cancer cells is conferred by overexpression of the human ABC transporter MDR1 or P-glycoprotein. Therefore, our research focused on a search for ABC-transporter homologues in the *A. nidulans* genome. These studies resulted in the characterization of the first two ABC-transporter genes (*atrA* and *atrB*) of the ATP-binding cassette superfamily from a filamentous fungus (Chapter 2). In addition, we report the characterization of five additional ABC-transporter-encoding (*atrC-atrG*) genes from this fungus (Chapter 3 and 5). By now, the superfamily of ABC transporters has more than a thousand members identified. Hence,

it comprises the largest protein family known to date, and many additional *atr* genes are expected to occur in the genome of *A. nidulans*.

The role of the identified *atr* genes in MDR was studied by expression analysis after drug treatment and by assessing the sensitivity of genetically-engineered deletion and overexpression mutants of *atr* genes (Chapters 2-6). Expression of *atr* genes was also analyzed in the *ima* mutants of *A. nidulans* (Chapter 5). Biochemical experiments confirmed that altered sensitivity to fungicides observed in the *atr* deletion and overexpression mutants can be ascribed to differential accumulation of the compounds in fungal mycelium (Chapters 3, 4 and 6).

Attention was also focussed on a putative role of *atr* genes in secretion of endogenous secondary metabolites. More specifically, we have tested the hypothesis whether *atr* genes play a role in penicillin production (Chapter 3).

In Chapter 7, the results obtained in our studies and relevant aspects that may apply to other filamentous fungi are discussed.

Chapter 1

ABC transporters and their impact on pathogenesis and drug sensitivity

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SUMMARY

This review presents an outline of the multifunctional properties of ABC transporters in different biological systems. A well-known function of these transport proteins is protection of organisms against toxic compounds. This also applies to plant pathogens. We propose that ABC transporters can play an important role in plant pathogenesis and fungicide sensitivity and thus can be regarded as potential target sites for the discovery of new biologically active compounds.

INTRODUCTION

Transport is one of the most important and fascinating aspects of life and an essential requirement in all organisms. Unicellular organisms need to maintain their homeostatic balance with constant uptake and allocation of nutrients and the secretion of toxic (waste) products. They must also be able to sense changes in their biotic and abiotic environment. In addition, multicellular organisms need to transport metabolites and information to and from organs. Multicellular organisms even possess specialized organs (tissues) for transport functions, *e.g.*, the blood and nervous system in animals and the vascular tissue in plants.

The main barrier for any transport event is the plasma membrane. Compounds can passively cross this barrier by diffusion. Transport by diffusion is possible only down a concentration gradient and is limited to solutes able to partition in hydrophobic membranes. Therefore, transport of most compounds over membranes is mediated by membrane bound proteins with specialized transport functions. With the unraveling of the genomes from different organisms the importance of membrane transporters becomes obvious. For instance, the complete genomic sequence of the gram-positive bacterium *Bacillus subtilis* possesses 2379 protein encoding ORFs with a known function. Of these proteins, 381 are likely to be involved in transport (Kunst *et al.*, 1997). This means that about 16% of the genes of this organism codes for membrane transporters. Several types of membrane transporter systems can be distinguished.

Ion Channels

Ion channels are membrane complexes mediating the movement of ions across plasma membranes as well as membranes of cell organelles. These channels form a pore allowing the passive flux of ions down its electrochemical gradient. The opening of

these channels is generally gated. This means that the opening is regulated by changes in membrane potential, membrane stretching or binding of a ligand. Ion channels play a role in diverse functions such as osmoregulation, cell growth, development, and nutrient uptake (Garrill *et al.*, 1993).

Facilitators

In contrast to ion channels, facilitators or carriers bind molecules to be transported and undergo a reversible change in conformation during transport. Based on the energy source driving the transport, facilitators can be classified in primary and secondary active transport systems.

Primary Active Transport Systems. Transporters belonging to this system couple transport to ATP hydrolysis. This provides the energy to transport solutes against an electrochemical gradient. Besides proton translocating ATPases two other families of ATP utilizing transporters are described. The P-type ATPases that make up a large superfamily of ATP-driven pumps involved in the transmembrane transport of charged substrates and the ATP binding cassette (ABC) transporters (Andre, 1995).

Secondary Active Transport Systems. Transporters belonging to this system derive the energy needed for transport from an electrochemical gradient over the membrane. Facilitated diffusion, the transport of solutes down its own electrochemical gradient, is generally mediated by uniporters. When transport of solutes takes place against an electrochemical gradient, the energy to drive this process is supplied by the symport or antiport of H^+ or other ions down their electrochemical gradient. A well characterized group is the major facilitator (MF) superfamily of transporters. Members of this superfamily function as H^+-substrate antiporters that use the proton motive force to drive transport (Fling *et al.*, 1991).

This review describes ABC transporters and presents an overview of their structural diversity and multifunctional character in a variety of biological systems. Emphasis will be on ABC transporters of (filamentous) fungi.

ABC TRANSPORTERS

Significance

ABC transporters are members of a large superfamily of transporters. Generally, they are located in plasma membranes and intracellular membranes and include both influx and efflux systems. ABC transporters are present from archae-bacteria to man but

became especially known for their involvement in multidrug resistance (MDR) in tumour cells (Higgins, 1992). The phenomenon of MDR is accompanied by a massive overproduction of ABC transporters (Beck, 1991). Besides MDR, they are also involved in various diseases such as cystic fibrosis, diabetes, adrenoleukodystrophy and the Zellweger syndrome. Furthermore, they play a role as peptide transporters in antigen presentation and in chloroquine resistance in the malarial parasite *Plasmodium falciparum* (Foote *et al.*, 1989; Lombard Platet *et al.*, 1996; Neefjes *et al.*, 1993).

Abundance of ABC Transporters

ABC transporter encoding genes are present in genomes of species representing all three domains of life *e.g.* archae, eubacteria and eukaryotae. In several of these classes of organisms, ABC transporters constitute the largest family of proteins (Table 1). Analysis of transport proteins in seven complete genomes of prokaryotic organisms shows that ABC-transporter and MF superfamilies account for an almost invariant fraction (0.38 to 0.53) of all transport systems per organism. The relative proportion of the two classes of transporters varies over a tenfold range, depending the organism (Paulsen *et al.*, 1998).

In eukaryotes the number of ABC transporters reported in literature is steadily increasing. In *Saccharomyces cerevisiae*, to date the only eukaryotic organism with the complete genome sequenced, 29 ABC-transporter proteins have been identified. In ongoing genome sequencing projects on other eukaryotic species, sequences homologous to ABC transporters have been detected as well.

Molecular Architecture

ABC-transporter proteins are characterized by the presence of several highly conserved amino acid sequences in their ABC domain. Two of these motifs, the Walker A [G-$(X)_4$-G-K-(T)-$(X)_6$-I/V] and Walker B [R/K-$(X)_3$-G-$(X)_3$-L-(hydrophobic)$_4$-D], are found in any ABC transporter and in many other proteins which bind and hydrolyze nucleotides (Ames *et al.*, 1990; Ames *et al.*, 1989; Bishop *et al.*, 1989; Walker *et al.*, 1982). The Walker motifs are separated by 120-170 amino acids including a motif characteristic for ABC transporters. This so-called ABC signature, [L-S-G-G-$(X)_3$-R-hydrophobic-X-hydrophobic-A] is highly conserved among ABC transporters only (Croop, 1993). The presence of multiple membrane spanning regions is also characteristic for ABC transporters.

Table 1. Number of ABC transporters in species representing different domains of life

Domain	Category	Species	Genome size (nt)	ABC transporters [a]	Ref.
Archae	Euryarchaeotae	*Archaeoglobus fulgidus*	2,178,400	40	(Klenk *et al.*, 1997)
Eubacteria	Firmicutes	*Bacillus subtilis*	4,214,807	77	(Kunst *et al.*, 1997)
Eubacteria	Proteobacteria	*Escherichia coli*	4,639,221	79	(Blattner *et al.*, 1997)
Eukaryotae	Fungi	*Saccharomyces cerevisiae*	12,069,313	29	(Decottignies and Goffeau, 1997)

[a] The figures do not give the number of transport systems, since these can be assembled from different polypeptides.

All members of the ABC-transporter superfamily have a modular architecture. The majority of the ABC transporters in higher organisms consists of two transmembrane domains (TMD), each with six predicted membrane spanning regions, and two intracellular located nucleotide binding folds (NBF) in a two times two-domain configuration. The nucleotide-binding domain can be either located at the amino terminus or at the carboxy terminus of the polypeptide, yielding proteins with a [TMD-NBF]$_2$ or [NBF-TMD]$_2$ configuration. The best characterized examples of ABC transporters with the [NBF-TMD$_6$]$_2$ and [TMD$_6$-NBF]$_2$ configuration are the yeast multidrug transporter Pdr5p and the human multidrug transporter P-glycoprotein (P-gp or MDR1), respectively (Balzi *et al.*, 1994; Endicott and Ling, 1989; Gottesman *et al.*, 1995; Juliano and Ling, 1976).

The domains can be formed as separate polypeptides or as a single polypeptide with one or more domains fused. Separate polypeptides subsequently aggregate to form functional transporters. In eukaryotic organisms the polypeptides are generally composed of at least two domains but usually contain all four domains (Blight and Holland, 1990). The so-called "half-sized" transporters with a [TMD-NBF] or [NBF-TMD] configuration are likely to function as dimers (Shani and Valle, 1996).

Multidrug resistance associated proteins (MRP) form a subfamily of ABC transporters with a TMD$_4$-[TMD$_6$-NBF]$_2$ topology. The main difference with other ABC transporters resides in the presence of an additional transmembrane spanning region at the protein amino terminus. An additional difference is the presence of the so-

called R-region located between the two homologous halves. The R-region is involved in regulation of the protein. MRPs act as glutathione-S-conjugate carriers and have been identified in a broad variety of organisms. The best-described example is the human MRP involved in broad-spectrum drug resistance (Broeks *et al.*, 1996; Cole *et al.*, 1992; Cui *et al.*, 1996).

Substrates

Substrates of ABC transporters range from 107 kDa proteins (*e.g.* haemolysin) to ions (*e.g.* Cl^-) (Anderson *et al.*, 1991; Blight and Holland, 1990). Most of the mammalian MDR proteins are (by definition) able to transport a wide variety of compounds although substrate specific transporters also occur. For instance, the human ABC-transporter P-gp (MDR1) has 93 known substrates from various chemical classes either of natural or synthetic origin. The main denominator is their high hydrophobicity (Gottesman and Pastan, 1993). Recently, a screening of the structures of these 93 substrates for potential spatial relationships between structural elements responsible for interaction with P-gp revealed that the presence of two or three electron donor groups with a spatial separation of 2.5 or 4.6 Å could be correlated with interaction with P-gp (Seelig, 1998).

Eukaryotic organisms also contain ABC transporters with a specific substrate range. For example, Ste6p from *S. cerevisiae* involved in secretion of the mating-factor pheromone and TAP1 and TAP2 involved in human antigen presentation (Kuchler *et al.*, 1989; Shepherd *et al.*, 1993). Bacterial ABC transporters involved in drug resistance have a very specific substrate specificity and are known as specific drug resistance transporters (SDR). Only one bacterial ABC transporter involved in MDR, LmrAp from *Lactococcus lactis*, has been detected (Bolhuis *et al.*, 1996b; Van Veen *et al.*, 1998).

Although ABC transporters are generally described as transporters some can also act as channels and regulators of channels. The cystic fibrosis transmembrane conductance regulator (CFTR) is an ABC transporter with channel function. The associated chloride channel is time and voltage independent and requires ATP hydrolysis for opening (Bear *et al.*, 1992; Riordan *et al.*, 1989). The human P-gp seems to control an associated ATP-dependent volume regulated chloride channel activity (Hardy *et al.*, 1995; Valverde *et al.*, 1996).

P-GLYCOPROTEIN

The human P-gp (MDR1) is probably the best characterized ABC transporter involved in multidrug resistance. Detailed structure-function relationship studies have been performed and its structure has been determined to 2.5-nm resolution (Rosenberg *et al.*, 1997). P-gp was first described in hamster cell lines in which the MDR phenomenon was correlated with the overexpression of a 170 kDa protein (Juliano and Ling, 1976). In human, two P-gp homologues, MDR1 and MDR3, have been identified. MDR1 is involved in broad-spectrum drug resistance and MDR3 in the translocation of phosphatidylcholine (Van Helvoort *et al.*, 1996). The overexpression of MDR1 in resistant cells with a low and high degree of resistance is due to elevated mRNA levels caused by regulatory mutations and gene amplification, respectively (Gudkov, 1991).

Catalytic Sites

Biochemical evidence and amino acid sequence information suggest that P-gp has ATPase activity. Membrane bound or purified P-gp preparations show a basal ATPase activity which can be stimulated by several drugs (al-Shawi and Senior, 1993). Both nucleotide binding folds bind and hydrolyze ATP (al-Shawi *et al.*, 1994). Synthetic half-sized P-gp molecules also display basal ATPase activity. However, interaction between both halves seems necessary for stimulation of ATPase activity by drugs (Loo and Clarke, 1994). This is also demonstrated by mutating either of the two-nucleotide binding domains. Inactivation of NBF_1 results in a block of ATP hydrolysis in NBF_2 and abolishes the drug extrusion capacity of the cells, and *vice versa* (Loo and Clarke, 1995a). Interaction between nucleotide binding sites was also demonstrated by vanadate-trapping experiments. This inhibitor of ATPase activity traps ADP in a catalytic site and trapping of ADP at only one site is sufficient to block ATPase activity of the entire protein (Urbatsch *et al.*, 1995a, 1995b).

These results have lead to a model for the catalysis mediated by P-gp in which both catalytic sites alternately undergo ATP hydrolysis. ATP binding at one site promotes ATP hydrolysis at the other. This induces a conformational change preventing the hydrolysis of the new-bound ATP. This new conformation has a high energetic state and relaxation of this conformation leads to the release of ADP and P_i, and transport of a substrate (Senior *et al.*, 1995).

Substrate Binding and Transport

Photoaffinity labeling and mutant analyses indicate that both membrane-bound halves of ABC transporters are involved in substrate binding. The substrate binding sites are located at the cytoplasmic site of the membrane, especially in transmembrane loops 4, 5, 6, 10, 11 and 12 (Greenberger, 1993; Loo and Clarke, 1995b; Safa *et al.*, 1990; Zhang *et al.*, 1995).

The way ABC transporters expel their substrates is not completely understood. ABC transporters probably act as "hydrophobic membrane cleaners" by detecting drugs which partition in membranes because of their hydrophobic nature. The possibility that transport out of the cytosol also contributes to the efflux can not be excluded (Bolhuis *et al.*, 1996a, 1996b; Gottesman and Pastan, 1988). The result of both transport processes is reduced accumulation of toxic compounds at their intracellular target site. The recently determined structure of P-gp revealed a large central pore forming a chamber within the membrane (Rosenberg *et al.*, 1997). Whether this pore is involved in the transport process or whether the transport occurs through conformational changes upon ATP-binding and hydrolysis remains unclear.

PHYSIOLOGICAL FUNCTIONS

Prokaryotes

Bacterial ABC transporters can be functionally grouped in two major distinct subfamilies. The superfamily of importers is responsible for transport of nutritional substrates. These transporters are also called periplasmic permeases and have a multisubunit component system with a similar structural organization (Doige and Ames, 1993). The presence of a periplasmic binding protein and the synthesis of the import system subunits (NBF and TMD) as separate polypeptides are distinctive features to the eukaryotic ABC proteins. The histidine permease from *Salmonella typhimurium* is a well characterized member of this subfamily. It is composed of the histidine-binding protein (HisJp) as the receptor, and the membrane-bound complex formed by two copies of HisPp (NBF) plus the HisQp and HisMp (TMD) (Kerppola *et al.*, 1991).

The subfamily of ABC exporters is involved in secretion of proteins, peptides and non-proteinaceus compounds (Fath and Kolter, 1993). In general, a basic functional structure for ABC exporters is composed of dimeric molecules (Wandersman, 1998). The ATP-binding motif of this subfamily shows a higher degree of similarity with the eukaryotic ABC proteins as compared to the above mentioned ABC importers. In addition,

some ABC exporters have their NBF and TMD domains synthesized as a single polypeptide. In gram-negative bacteria, additional export proteins are required for transport to the extracellular medium. For instance, HlyDp and TolCp which are involved in the secretion of haemolysin in *E. coli* (Wandersman and Delepelaire, 1990). Other examples of prokaryotic ABC transporters are the export system of proteases A, B and C in the phytopathogenic bacterium *Erwinia chrysanthemi* (Letoffe *et al.*, 1990), the secretion machinery of peptide antibiotics (bacteriocins) from *Lactococcus lactis* (Stoddard *et al.*, 1992), and the β-1,2-glucans oligomers export systems of the plant pathogen *Agrobacterium tumefaciens* (ChvAp) (Cangelosi *et al.*, 1989), and the symbiont *Rhizobium meliloti* (NdvAp) (Stanfield *et al.*, 1988). β-1,2-glucans oligomers are involved in the attachment of the bacteria to plant cells. Therefore, ChvAp can be regarded as a virulence and NdvAp as a nodulation factor.

Eukaryotes

Yeasts. With the unraveling of the complete genome sequence of *S. cerevisiae* 29 ABC proteins were identified by sequence homology (Decottignies and Goffeau, 1997). Only ten of these proteins have a known physiological function. The 29 encoded ABC polypeptide sequences could be divided in six subfamilies. The majority of the proteins have the tetra-domain modular architecture comprising nine proteins with the [NBF-TMD]$_2$ and seven with the reverse [TMD-NBF]$_2$ topology. Furthermore, "half-sized" [TMD-NBF] proteins, which likely function as dimers, were detected. For instance, the peroxisomal ABC transporters Pxa1p and Pxa2p form heterodimers and are involved in long-chain fatty acid transport and β-oxidation (Shani and Valle, 1996). The yeast ABC proteins with a known physiological function, different from a role in MDR, are listed in Table 2. The MDR proteins of *S. cerevisiae* are discussed bellow, in the section multidrug resistance.

In the fission yeast *Schizosaccharomyces pombe* and the human pathogen *Candida albicans*, ABC proteins have also functionally been described. All of them have an orthologue in the genome of *S. cerevisae* (Balan *et al.*, 1997; Christensen *et al.*, 1997; Ortiz *et al.*, 1992; Ortiz *et al.*, 1995; Raymond *et al.*, 1998).

Filamentous Fungi. Members of the ABC-transporter superfamily have been described for at least seven fungal species (Table 3). The saprophyte *Aspergillus nidulans*, the human-pathogens *Aspergillus flavus* and *Aspergillus fumigatus* and the plant pathogens

Magnaporthe grisea (rice blast), *Botryotinia fuckeliana* (grey mould), *Mycosphaerella graminicola* (wheat leaf blotch) and *Penicillium digitatum* (citrus green mould) (Del Sorbo *et al.*, 1997; Nakaune *et al.*, 1998; Schoonbeek, 1998; Tobin *et al.*, 1997; Zwiers, 1998).

Table 2. ABC transporters from *Saccharomyces cerevisiae* with an identified physiological function

Gene	GenBank number	Size (aa)	TMD	Topology	Knock-out	Function	Ref.
STE6	Z28209	1290	12	$[TMD + NBF]_2$	Viable	a-pheromone export	(Kuchler *et al.*, 1989)
ATM1	Z49212	690	5	TMD + NBF	Restricted growth	Mitochondrial DNA maintenance	(Leighton, 1995)
PXA1	L38491	870	5	TMD + NBF	Viable	VLCFA beta-oxidation	(Hettema *et al.*, 1996)
PXA2	X74151	853	6	TMD + NBF	Viable	Interaction with PXA1	(Hettema *et al.*, 1996)
GCN20	D50617	752	0	$[NBF]_2$	Viable	Interactions with tRNA and GCN2	(Vazquez de Aldana *et al.*, 1995)
YEF3	U20865	1044	3	$[NBF]_2$	No growth	Aminoacyl-tRNA binding to ribosomes	(Sandbaken *et al.*, 1990)

In addition, many other members are expected to be revealed in ongoing fungal genome sequencing projects. We screened available expressed sequence tags (EST) databases of *A. nidulans* and *N. crassa* for potential homologues of ABC transporters with the conserved motifs listed in Table 3 (Nelson *et al.*, 1997; Roe *et al.*, 1998). The search was performed with the BLAST program of sequence alignment and yielded seven homologous sequences from *A. nidulans* and two from *N. crassa* (Table 4) (Altschul *et al.*, 1997). EST clones identical to *atrC* and *atrD*, two previously characterized genes from *A. nidulans*, were also detected (Andrade *et al.*, 1998).

The physiological relevance of ABC transporters in filamentous fungi is probably high (De Waard, 1997). For instance, a number of them may be involved in secretion of secondary metabolites, which in the case of fungitoxic compounds, can act as a self-protection mechanism. Similarly, ABC transporters may provide protection against toxic metabolites produced by other microorganisms present in particular ecosystems. Plant pathogenic fungi have to cope with a variety of plant defense compounds and they may possess ABC transporters that function in protection against the toxic action of such compounds as well. These hypotheses are supported by the observation that a wide variety

of natural compounds such as isoflavonoids, plant alkaloids and antibiotics can act as substrates of ABC transporters (Gottesman and Pastan, 1993; Seelig, 1998). In addition, specific ABC transporters of filamentous fungi may function in secretion of a mating factor as shown for several yeast species. Therefore, ABC transporters can mediate processes important for survival of fungi in nature and hence, may function as significant parameters in the population dynamics of these organisms.

Table 3. Multiple alignment of conserved sequences from reported ABC-transporter proteins from filamentous fungi

Species	Gene	GenBank number	Domain		
				N terminal	
			Walker A	ABC signature	Walker B
A. nidulans	*atrA*	Z68904	LGRPGTGCSTFL	VSGGERKRVSIAE	AAWDNSSRGLD
A. nidulans	*atrB*	Z68905	LGRPGSGCTTLL	VSGGERKRVSIIE	FCWDNSTRGLD
B. fuckeliana	*pgp1*	Z68906	LGRPGSGCSTFL	VSGGERKRVSIAE	VSWDNSTRGLD
M. grisea	*abc1*	AF032443	LGPPGSGCSTFL	VSGGERKRVTIAE	QCWDNSTRGLD
A. fumigatus	*mdr1*	U62934	VGPSGSGKSTVV	LSGGQKQRIAIAR	LLLDEATSALD
A. flavus	*mdr1*	U62932	VGPSGSGKSTII	LSGGQKQRIAIAR	LLLDEATSALD
			* * * *	*** * *	* **
				C terminal	
			Walker A	ABC signature	Walker B
A. nidulans	*atrA*	Z68904	MGVSGAGKTTLL	LNVEQRKLLTIGV	LFLDEPTSGLD
A. nidulans	*atrB*	Z68905	MGSSGAGKTTLL	LSVEQRKRVTIGV	IFLDEPTSGLD
B. fuckeliana	*pgp1*	Z68906	MGASGAGKTTLL	LSVEQRKRVTIGV	LFLDEATSGLD
M. grisea	*abc1*	AF032443	MGVSGAGKTTLL	LNVEQRKRLTIGV	LFVDEPTSGLD
A. fumigatus	*mdr1*	U62934	VGPSGCGKSTTI	LSGGQKQRVAIAR	LLLDEATSALD
A. flavus	*mdr1*	U62932	VGASGSGKSTTI	LSGGQKQRIAIAR	LLLDEATSALD
A. fumigatus	*mdr2*	U62936	VGPSGGGKSTIA	LSGGQKQRIAIAR	LILDEATSALD
			* ** ** *	* * *	** ** **

Asterisks indicate identical amino acid residues.

Table 4. Partial sequences of putative ABC transporters detected in the Expressed Sequence Tags (EST) databases from *Aspergillus nidulans* (A.n.) and *Neurospora crassa* (N.c.)

Species	*EST clone*	*GenBank number*	*Walker A*	*ABC signature*	*Walker B*	*S. cerevisae homologue*	*BLAST score* [a]
A.n.	h8h04a1.r1	AA785885		NVEQRKRLTIGV	LFLDEPTSGLD	Pdr10p	e^{-47}
A.n.	c9e04a1.f1	AA783966		SGGQKQRLCIAR	LLLDEATSSLD	Mdl1p	e^{-17}
A.n.	o8f05a1.f1	AA787659			LLLDESTSALD	Ycf1p	e^{-09}
A.n.	e7d04a1.r1	AA784517	GPNGSGKTTLM			Yef3Bp	e^{-30}
A.n.	m7a02a1.r1	AA786886	GRNGAGKSTLM			YPL226w	e^{-23}
A.n.	e4a06a1.r1	AA784449	GLNGCGKSTLI			YPL226w	e^{-07}
A.n.	k5a05a1.f1	AA786673			SFLDEPTNTVD	Yef3Bp	e^{-44}
N.c.	NCM8C11T7	AA901957		SQGQRQLVGLGR	VIMDEATASID	YLL015w	e^{-20}
N.c.	NCC3E5T7	AA901865		SDGQKSRIVFAL	LLLDEPTNGLD	YER036c	e^{-55}

[a] Based on homology of the full EST clone.

Higher eukaryotes. Basically, the majority of the ABC transporters characterized in higher eukaryotes have an orthologue in the *S. cerevisae* genome or at least a very close homologue with similar substrate specificity. However, due to evolutionary speciation, physiological needs may be different and account for differences in ABC-transporter proteins. This is well illustrated by the high number of MRP-like transporters already characterized in the genome of *Arabidopsis thaliana* (Tommasini *et al.*, 1997). These transporters share with Ycf1p, the closest yeast homologue, glutathione-S-conjugate transport activity. In addition, plant MRP proteins have the property to transport chlorophyll catabolites (Lu *et al.*, 1998; Tommasini *et al.*, 1998). Other physiological functions of ABC transporters in higher eukaryotes have been described as well (Anderson *et al.*, 1991; Broeks *et al.*, 1996; Luciani and Chimini, 1996; Paulusma *et al.*, 1996; Ruetz and Gros, 1994; Van Helvoort *et al.*, 1996).

MULTIDRUG RESISTANCE

The use of cytotoxic compounds such as drugs in clinical medicine and disease control agents in agriculture is an essential component of human life. However, the widespread and sometimes excessive use of these compounds has resulted in a high selection pressure resulting in drug resistant populations. This phenomenon is of major concern to society.

In general, the major mechanisms underling resistance in prokaryotes and eukaryotes can be classified as follows: (a) enzymatic inactivation or degradation of drugs, (b) alterations of the drug target-site and, (c) decreased drug-accumulation caused by energy-dependent drug efflux. More than one mechanism may operate in concert and the sum of different alterations represents the final resistant phenotype.

In several cases the resistance mechanism not only conferred decreased sensitivity to a specific drug (and analogues) used during the selection process, but also to several structurally and functionally unrelated compounds. This phenomenon, termed multidrug resistance (MDR) has been described to operate in a broad range of organisms. It relates to decreased accumulation of drugs via energy-dependent drug efflux systems. The majority of the transport proteins involved in drug-extrusion as determinants of MDR belong either to the ABC transporter or the MF superfamilies.

ABC Transporters and MDR in Prokaryotes and Lower Eukaryotes

In prokaryotes most of the characterized efflux-systems involved in MDR utilize the proton motive force as energy source for transport and act via a drug/H^+ antiport mechanism. The first example of a prokaryotic ABC transporter involved in MDR is the LmrAp protein from Lactococcus *lactis* (Van Veen *et al.*, 1996). The gene encodes a 590 aa membrane protein with the TMD_6-NBF topology. The protein probably functions as a homodimer. Functional studies performed in *E. coli* indicate that its substrate specificity comprises a wide range of hydrophobic cationic compounds, very similar to the pattern displayed by the human MDR1. Surprisingly, when expressed in human lung fibroblast cells, LmrAp was targeted to the plasma membrane and also conferred typical multidrug resistance, confirming the evolutionary relation of these two proteins (Van Veen *et al.*, 1998).

Genes encoding ABC transporters in parasitic protozoa have been isolated and analyzed from *Plasmodium, Leishmania*, and *Entamoeba spp.*, and variation in the copy-number and/or levels of expression have been implicated in drug resistance (Ullman, 1995).

From *S. cerevisiae*, at least four members of the ABC transporter superfamily are involved in MDR: Pdr5p, Snq2p, Ycf1p and Yor1p (Balzi *et al.*, 1994; Cui *et al.*, 1996; Decottignies *et al.*, 1995; Li *et al.*, 1997). Pdr5p and Snq2p have the $[NBF\text{-}TMD]_2$ topology and preferential substrate specificity for aromatic cationic compounds, whereas Ycf1p and Yor1p have the $[TMD\text{-}NBF]_2$ orientation and substrate specificity for anionic

compounds. Despite its inverted topology and low sequence similarity, Pdr5p seems to be the yeast functional homologue of the human MDR1, if substrate specificity is considered (Kolaczkowski *et al.*, 1996). The presence of several other ABC proteins from *S. cerevisiae* with high homology to the ones involved in MDR and with common regulatory mechanisms suggests that other ABC transporters may be involved in MDR of *S. cerevisiae* as well (Balzi and Goffeau, 1995). In yeast species such as *S. pombe* and *C. albicans* multidrug-efflux systems based on overproduction of ABC transporters have also been identified. Examples are Cdr1p and Cdr2p from *C. albicans* and Pmd1p and Bfr1p from *S. pombe* (Nagao *et al.*, 1995; Nishi *et al.*, 1992; Sanglard *et al.*, 1996, 1997).

MDR in Filamentous Fungi

MDR in filamentous fungi has been reported for laboratory generated mutants of *A. nidulans* selected for resistance to azole fungicides. In genetically defined mutants, resistance to azoles is based on an energy-dependent efflux mechanism, which results in decreased accumulation of the compounds in fungal mycelium (De Waard, 1995). This mechanism also operates in other species such as *P. italicum*, *B. fuckeliana, Nectria haematococca* and probably *M. graminicola* (De Waard, 1995; Joseph-horne *et al.*, 1996). In our laboratory, ABC transporter encoding genes from *A. nidulans*, *B. fuckeliana* and *M. graminicola*, have been isolated and are currently functionally characterized (Andrade *et al.*, 1998; Del Sorbo *et al.*, 1997; Schoonbeek, 1998; Zwiers, 1998). The isolated genes display a high degree of homology with Pdr5p and Pmd1p, yeast ABC transporters involved in MDR. AtrBp from *A. nidulans* complements a Pdr5p null mutant of *S. cerevisiae*, suggesting indeed a role in fungicide sensitivity and resistance.

Very recently, the involvement of an ABC transporter (Pmr1p) in azole resistance has been established for field isolates of the phytopathogenic fungus *P. digitatum*, the causal agent of citrus green mold (Nakaune *et al.*, 1998). Another example is AfuMdr1p from the human pathogen *A. fumigatus* which confers decreased sensitivity to the antifungal compound cilofungin when overexpressed in yeast (Tobin *et al.*, 1997).

EVOLUTIONARY ASPECTS OF ABC TRANSPORTERS

The ubiquitous occurrence of ABC transporters throughout the living world indicates the ancient character of this superfamily of proteins. They are believed to date back in evolutionary time for more than 3 billion years (Saier *et al.*, 1998). Thus, the understanding of evolutionary relationships among these transporters might be helpful in

elucidating the origins of multidrug efflux systems, their physiological functions, and more important, the nature of their substrate specificity.

Recently, two paradigms on the evolution of bacterial multidrug transporters have been proposed. The first one describes that the transporters have evolved to protect cells from structurally diverse environmental toxins. The second one states that the transporters initially functioned in transport of specific physiological compounds (or a group of structurally related natural compounds) with the ability to expel drugs being only a fortuitous side effect (Neyfakh, 1997). Experimental evidence has been proposed for both hypotheses, but it is unlikely that transport proteins have evolved numerous distinct binding sites for structurally dissimilar molecules and therefore, a physiological substrate is likely to exist (Poole, 1997). Furthermore, the presence of accessory factors as determinants of substrate specificity, such as the periplasmic binding proteins of the prokaryotic uptake systems or the eukaryotic glutathione-S-conjugate export pumps, could explain, in part, the accommodation of structurally unrelated compounds by ABC proteins.

Comparison of multidrug transport systems from six complete genomes of bacteria (three pathogenic and three non-pathogenic), indicates that, with one exception (*Methanococcus jannaschii*), the number of multidrug-efflux pumps is approximately proportional to the total number of encoded transport systems as well as the total genome size. Therefore, the similar numbers of chromosomally encoded multidrug efflux systems in pathogens and nonpathogens suggest that these transporters have not arisen recently in pathogenic isolates in response to antimicrobial chemotherapy (Saier *et al.*, 1998). However, during speciation, novel ABC-transporter proteins with modified substrate specificity might have evolved, as a result of fusions, intragenic splicings, duplications and deletions, in order to accomplish the different needs of organisms occurring in distinct environments. This can be illustrated by the occurrence of bacterial ABC transporters as separate subunits (*e.g.*, NBF and TMD) and by the inverted topology of domains observed in eukaryotic proteins. In addition, neither homologous proteins nor a characteristic motif of the so-called cluster I of yeast ABC transporters have, as yet, been found in prokaryotes (Decottignies and Goffeau, 1997).

The considerations mentioned above and the experimental data available suggest that a MDR phenotype is not primarily caused by the appearance of a novel transport protein with a modified substrate profile but rather by an increased expression level of a pre-existent transport system as a result of alterations in regulation of such proteins (PDR in yeast) or gene amplification (MDR in mammalian).

PERSPECTIVES

Since the early 1980's the significance of ABC transporters for drug sensitivity and resistance has been recognized in the medical field. A similar interest in the role of ABC transporters in agriculture only started recently. Now, there is a growing awareness that ABC transporters can be involved in mechanisms of natural insensitivity and acquired resistance in a wide range of organisms. In this review, we provide evidence that this also holds true for (pathogenic) filamentous fungi.

In the treatment of MDR-cancer cells, inhibitors of ABC-transporter activity are used as synergists of drugs to reduce the MDR phenotype. If MDR would be the main mechanism of resistance to azoles, similar inhibitors could be useful in mixtures with these fungicides to increase control of azole resistant populations of (plant) pathogenic fungi. If ABC transporters also play a role in protection against plant defense compounds and/or secretion of pathogenicity factors, inhibition of ABC transporter activity would result in enhanced host resistance and/or reduced virulence of the pathogen. Both processes would reduce disease development. As described for *S. cerevisiae* and *S. pombe* specific ABC transporters can be responsible for the transport of a mating factors (Christensen *et al.*, 1997; McGrath and Varshavsky, 1989). Inhibition of the activity of such specific ABC transporters would prevent mating, reduce the genetic variation and retard the epidemiology of plant pathogenic fungi.

In *S. cerevisiae* and *C. albicans,* ABC transporters with an $[NBF]_2$ configuration have been described. These so-called cluster IV ABC transporters interact with tRNA and act as elongation factors. ABC proteins of this cluster are interesting target sites for antifungal compounds as they seem to be absent from mammals (Decottignies and Goffeau, 1997; Vazquez de Aldana *et al.*, 1995).

Although evidence is accumulating that fungal ABC transporters are involved in pathogenesis and (fungicide) resistance, more research is needed to assess the full significance of ABC transporters in these phenomena. Knock-out mutants and mutants overexpressing ABC transporters will help gaining insight in the physiological functions of ABC-transporters. Knock-out mutants lacking the natural insensitivity provided by ABC transporters can also be used as tools to screen for compounds with intrinsic fungitoxic activity.

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Chapter 2

Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters

G. Del Sorbo, A.C. Andrade, J.G.M. Van Nistelrooy, J.A.L. Van Kan, E. Balzi and M.A. De Waard. *Mol Gen Genet* (1997) **254**: 417-426.

SUMMARY

Two single-copy genes, designated *atrA* and *atrB* (ATP-binding cassette transporter A and B), were cloned from the filamentous fungus *Aspergillus nidulans* and sequenced. Based on the presence of conserved motifs and on hydropathy analysis, the products encoded by *atrA* and *atrB* can be regarded as novel members of the ATP-binding cassette (ABC) superfamily of membrane transporters. Both products share the same topology as the ABC transporters Pdr5p and Snq2p from *Saccharomyces cerevisiae* and Cdr1p from *Candida albicans*, which are involved in multidrug resistance of these yeasts. Significant homology also occurs between the ATP-binding cassettes of AtrAp and AtrBp, and those of mammalian ABC transporters (P-glycoproteins). The transcription of *atrA* and, in particular, *atrB* in mycelium of *A. nidulans* is strongly enhanced by treatment with several drugs, including antibiotics, azole fungicides and plant defense toxins. The enhanced transcription is detectable within a few minutes after drug treatment and coincides with the beginning of energy-dependent drug efflux activity, reported previously in the fungus for azole fungicides. Transcription of the *atr* genes has been studied in a wild type and in a series of isogenic strains carrying the *imaA* and/or *imaB* mutations, which confer multidrug resistance to various toxic compounds such as the azole fungicide imazalil. *atrB* is constitutively transcribed at a low level in the wild type and in strains carrying *imaA* or *imaB*. Imazalil treatment enhances transcription of *atrB* to a similar extent in all strains tested. *atrA*, unlike *atrB*, displays a relatively high level of constitutive expression in mutants carrying *imaB*. Imazalil enhances transcription of *atrA* more strongly in *imaB* mutants, suggesting that the *imaB* locus regulates *atrA*. Functional analysis demonstrated that cDNA of *atrB* can complement the drug hypersensitivity associated with *PDR5* deficiency in *S. cerevisiae*.

INTRODUCTION

Resistance to multiple chemically unrelated drugs is a general phenomenon described in both prokaryotes (Lewis, 1994) and eukaryotes (Higgins, 1992). This phenomenon is referred to as multidrug resistance (MDR). MDR can be caused by increased ATP-dependent efflux of toxic compounds from the cytoplasm and plasma membrane, mediated by membrane-bound ATP-dependent transporters of the ABC (**A**TP-**B**inding **C**assette) superfamily (reviews by Balzi and Goffeau, 1995; Gottesman and Pastan, 1993; Juranka *et al.*, 1989; Schinkel and Borst, 1991; Ullman, 1995). Increased transcription of the mammalian gene *MDR1*, usually accompanied by gene amplification, is generally observed in tumour cell lines displaying

MDR to antitumour drugs. Yeast genes encoding another subfamily of ABC transporters, such as *PDR5* (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994; Hirata *et al.*, 1994) and *SNQ2* (Servos *et al.*, 1993), can also confer a multidrug resistance phenotype when introduced into cells in multiple copies on plasmids (Haase *et al.*, 1992; Leppert *et al.*, 1990), or upon point mutation of the regulatory loci *PDR1* and *PDR3* (Balzi and Goffeau, 1994, 1995). Yeast proteins Snq2p and Pdr5p display a domain topology different from the mammalian protein. They also share sequence peculiarities in the nucleotide-binding domain, such as degenerated nucleotide-binding motifs (Balzi *et al.*, 1994). Despite these structural differences, the yeast Pdr5p and Snq2p share with the mammalian MDR1 protein many biochemical features and functions of drug transport coupled to hydrolysis of nucleotide triphosphates (Decottignies *et al.*, 1994, 1995).

The topology of the mammalian MDR1 gene product, also known as P-glycoprotein (Juliano and Ling, 1976) is characterized by two membrane-anchored hydrophobic moieties with six transmembrane stretches, alternating with two intracellular ATP-binding hydrophilic moieties. The hydrophilic domains comprise a conserved ABC region, including the ATP-binding motifs known as Walker A and Walker B (Walker *et al.*, 1982) and another conserved motif, the so-called ABC signature, preceding the Walker B motif.

Mammalian P-glycoproteins are in general encoded by small gene families with two or three representatives in human and rodents, respectively, designated as *MDR* genes (Gottesman and Pastan, 1993). Although P-glycoproteins are best known for their role in MDR, some of them also function in secretion of non-toxic products (Smit *et al.*, 1993; Van Kalken *et al.*, 1993). This is also the case for the P-glycoprotein homologue Ste6p from *Saccharomyces cerevisiae*. This transporter mediates the secretion of the lipopeptide mating pheromone **a** and its deletion causes sterility (Kuchler *et al.*, 1989; McGrath and Varshavsky, 1989).

In addition to P-glycoproteins, another mammalian ABC transporter, the Multidrug Resistance-associated Protein (MRP), is also related to MDR (Cole *et al.*, 1992). In yeast, the ABC genes *YOR1* and *YCF1*, conferring resistance to oligomycin, cadmium, and diamid, respectively (Katzmann *et al.*, 1995; Szczypka *et al.*, 1994), most closely resemble MRP subfamily members in structure and function. Ycf1p, like MRP1, participates in the glutathione-dependent detoxification pathway by transporting glutathione S-conjugates (Li *et al.*, 1996).

Analysis of the complete yeast genome revealed the existence of 29 genes encoding ABC proteins displaying various combinations and dispositions of two to four hydrophobic

and hydrophilic domains (A. Decottignies and A. Goffeau, in preparation). On the basis of structural features such as domain composition and sequence homologies, it is possible to distinguish up to five different subclasses of ABC proteins (S. Michaelis and C. Berkower, in press; A. Decottignies and A. Goffeau, in preparation).

The phenomenon of MDR described for mammals and yeasts, also occurs in saprophytic and pathogenic filamentous fungi (De Waard *et al.*, 1996). Mutants of several fungal species selected for resistance to azole fungicides, which exert their toxic action by inhibition of the activity of sterol 14α-demethylase, displayed pleiotropic effects to many other, chemically unrelated compounds. Genetic determinants displaying pleiotropic effects have been identified and mapped (Van Tuyl, 1977). In *A. nidulans*, resistance to the azole fungicide imazalil can be determined by mutations in at least eight different genes (Van Tuyl, 1977). The mutations *imaA* (mapped on linkage group VII) and *imaB* (mapped on linkage group V) result in altered levels of resistance to other non-related toxicants, such as acriflavin and neomycin. The mechanism of drug resistance in these mutants depends on decreased accumulation caused by energy-dependent efflux of the azole fungicides from mycelium (De Waard and Van Nistelrooy, 1980), as described for drugs from multidrug-resistant tumour cells. As yet, none of these *ima* loci have been isolated.

The present paper reports the isolation, molecular cloning and functional analysis of two genes, *atrA* and *atrB* (ATP-binding cassette transporter A and B), encoding ABC transporters in the filamentous fungus *A. nidulans*. These are, to our knowledge, the first ABC superfamily members to be isolated that are probably involved in determining MDR in filamentous fungi. The two genes are differentially expressed in wild type and isogenic *ima* mutants after treatment with cycloheximide, the phytoalexin pisatin (a plant defense product of pea) and azole fungicides. The function of both genes has been analyzed by complementation of a *PDR5* null mutant of *S. cerevisiae* and characterization of the phenotype of the transformants. On the basis of these observations, we hypothesize that resistance in the *ima* mutants of *A. nidulans* may relate to activity of ABC transporters. This conclusion is of major interest for the understanding and management of resistance to azole antimycotics in mammalian pathogens such as *A. fumigatus*, the causal agent of systemic aspergillosis (Tobin *et al.*, 1996). Since this type of disease has steadily increased over the last ten years, especially in immunodepressed patients, this topic is of major concern. A comparable critical situation has been described for agriculture, where azole fungicides are widely used to control harmful plant pathogens in major agricultural crops (De Waard, 1994).

RESULTS

Phenotype characterization of multidrug resistant mutants

The pleiotropic drug resistance spectra of several isogenic *ima* mutants of *A. nidulans* were studied by determining their sensitivity to several unrelated chemicals in radial growth tests (Table 1). The results of our tests largely confirm those obtained by Van Tuyl (1977). *ima A* (strain 130) and *imaB* (strain 146) mutations confer similar levels of resistance to the azole and related fungicides imazalil, fenarimol and triadimenol, and their effects are additive (strain R264). *imaA* also confers resistance to neomycin and tomatine. *imaB* determines hypersensitivity to acriflavin and neomycin, and resistance to pisatin. The low degree of hypersensitivity to cycloheximide conferred by *imaB* described by Van Tuyl (1977), was not observed here. *ima A* and *imaB* do not alter significantly the level of resistance to other compounds tested (chlorpromazine, 4-nitroquinoline-*N*-oxide, oligomycin, triflupromazine, and valinomycin).

Table 1. Pleiotropic effects of isogenic imazalil-resistant mutant strains of *Aspergillus nidulans* in radial growth tests

Compound	EC_{50} (μg ml^{-1}) [a]			
	003 (wild-type)	130 (*imaA*)	146 (*imaB*)	264 (*imaA* + *imaB*)
Acriflavin	646 ± 8	607 ± 22 (0.91)	89 ± 4 (0.14)	137 ± 4 (0.21)
Fenarimol [b]	3.8 ± 0.2	8.2 ± 0.5 (2.2)	25.5 ± 0.9 (6.7)	27.3 ± 1.3 (7.2)
Imazalil [b]	0.08 ± 0.004	0.45 ± 0.02 (5.6)	0.86 ± 0.02 (10.7)	2.20 ± 0.02 (27.5)
Neomycin	1120 ± 20	2360 ± 30 (2.1)	288 ± 7 (0.2)	652 ± 14 (0.6)
Pisatin	23.1 ± 1.2	29.5 ± 2.2 (1.3)	108 ± 6 (4.7)	98.9 ± 6.1 (4.3)
Tomatine	68.2 ± 3.3	146 ± 4 (2.1)	54.5 ± 3.4 (0.8)	89.3 ± 4.1 (1.3)
Triadimenol [b]	13.5 ± 0.6	96.6 ± 2.3 (7.2)	101 ± 3 (7.5)	226 ± 6 (16.7)

[a] The EC50 values are given as means with standard deviations of three replicates. The resistance factor (EC50 mutant: EC50 wild type) is given in parentheses.
[b] Azole fungicides and related compounds that inhibit sterol biosynthesis.

Isolation and sequence analysis of the *atrA* and *atrB* genes

In order to isolate genes from *A. nidulans* conferring multiple drug resistance, we screened a non-amplified genomic library of *A. nidulans* with a probe derived from the region coding for

the conserved ABC cassette of the *PDR5* gene of *S. cerevisiae*. Two non-overlapping clones were isolated and designated as an1 and an2. Southern blot analysis revealed that both clones contained a hybridizing fragment, which could be detected in *A. nidulans* genomic DNA restricted with the same enzyme or combinations of enzymes (Fig. 1). The clones contained inserts of about 15.5 (an1) and 9.5 kb (an2), respectively (Fig. 2).

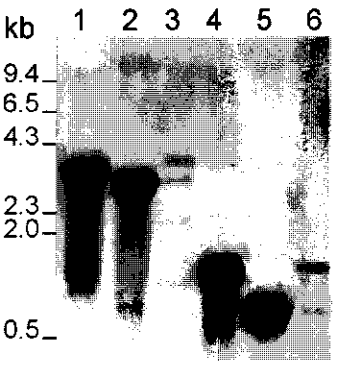

Figure 1. Southern blot analysis
Southern blot analysis of phage clones selected from a genomic library of *A. nidulans* by hybridization with a *PDR5* probe from *S. cerevisiae*. Lanes 1 and 4: clone an1 restricted with *Bam*HI and *Bam*HI + *Sal*I, respectively; lanes 2 and 5: clone an2 restricted with *Bam*HI and *Bam*HI + *Sal*I, respectively; lanes 3 and 6: genomic DNA from *A. nidulans* strain 003 restricted with *Bam*HI and *Bam*HI + *Sal*I. The probe used was a 1.52 kb *Bgl*II fragment spanning the N-terminal ATP-binding cassette of *PDR5*.

A 6.8 kb fragment of an1, containing the region that hybridized with the *PDR5* probe, was subcloned and entirely sequenced, starting 190 bp before the most upstream *Pst*I site (Fig. 2A). The sequence revealed the presence of an open reading frame (ORF) of 4398 bp, interrupted by four introns, ranging in size from 57 to 66 bp (Fig. 2B). The positions of introns were confirmed by sequencing of a cDNA. In the 5' flanking region several features of a promoter sequence were found. In particular, an adenine is present at position -3, a 9/10 conserved Kozak consensus (CCACC ATG GG) as found around the putative translation start. A TATA-like consensus (TATTAT) (Chen and Struhl, 1988), identical to that of the *amdS* gene of *A. nidulans*, is present at position -76. In addition, two 5/6 identical CAAT consensus sequences at positions -162 and -122, a heat shock element (HSE) consensus at position -706 and several other HSE-like (5/6 conserved) consensus sequences were found. In the 3' flanking region, two sequences 5/6 identical (+4653 AATACA and +5622 AATAGA) to the polyadenylation signal AATAAA (Proudfoot and Brownlee, 1976) were found 15 and

383 nucleotides downstream of the stop codon. The sequence is available in the EMBL database under the accession number Z68904.

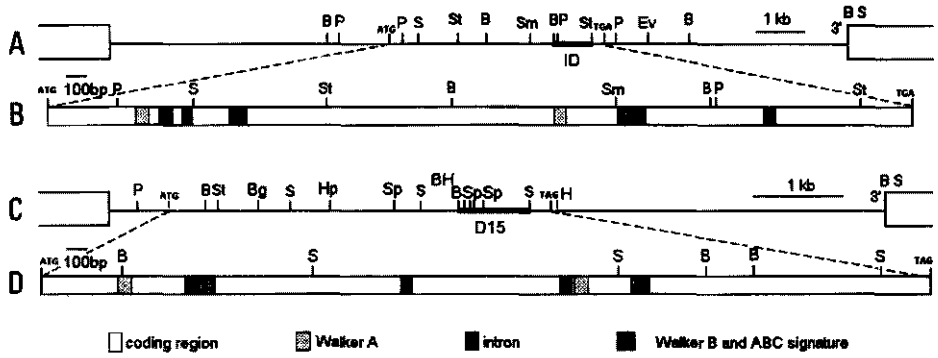

Figure 2. Physical map of isolated λ clones
Physical map of the inserts of phage an1 (**A**) and an2 (**C**) containing the ORFs of *atrA* (**B**) and *atrB* (**D**), respectively. Restriction sites are abbreviated as follows: B, *Bam*HI; P, *Pst*I; S, *Sal*I; St, *Sst*I; Sm, *Sma*I; Ev, *Eco*RV; Bg, *Bgl*II; Hp, *Hpa*I; Sp, *Sph*I; H, *Hind*III. Open boxes in **A** and **C** represent λ phage arms. ID (**A**) and D15 (**C**) represent the restriction fragments used as gene-specific probes. Putative translation and stop codons are indicated

The ORF of an1 encodes a protein of 1466 amino acids, with a calculated molecular weight of 162.6 kDa and an estimated isoeletric point of 6.45. The predicted structure of the protein is typical of an ABC superfamily member. Hydropathy analysis (Kyte and Doolittle, 1982; results not shown) revealed that each half of the protein consists of a N-terminal hydrophylic domain followed by a hydrophobic one. In the latter region several stretches of hydrophobic amino acids are present, thus suggesting the occurrence of transmembrane domains. The encoded protein has potential sites for N-glycosylation, phosphorylation, and myristylation.

An ATP-binding cassette is present in both halves of the protein. The cassette in the hydrophilic moiety of the N-terminal half consists of a degenerated Walker A motif (GRPGTGCS), a well-conserved ABC signature (VSGGERKRVSIA), and a degenerated Walker B motif (FAAWD). An ATP-binding cassette, with typical Walker A and B motifs but with a highly degenerated ABC signature is also present in the C-terminal hydrophylic moiety. The amino acid sequence of the protein is very similar to those of other members of the ABC superfamily. The best alignments were obtained with Pdr5p (45% identity and 64% similarity) and Snq2p (41% identity and 61% similarity) from *S. cerevisiae* and Cdr1p of *C. albicans* (45% identity and 65% similarity). The regions with the highest level of homology

are centered on the ABC cassettes. In view of the similarity of the protein encoded by the ORF present on the 6.8 kb fragment of an1 to other ABC transporters, the gene present on the 6.8 kb fragment of an1 was designated *atrA* and the encoded protein AtrAp.

```
N-TERMINAL DOMAIN          Walker A          ABC signature          Walker B
                           ________          _____________          ________
A. nidulans AtrAp          GELLLVLGRPGTGCSTFLKAV  VSGGERKRVSIAEMALAMTPFAAWDNSSRGLD
A. nidulans AtrBp          GEMLLVLGRPGSGCTTLLKML  VSGGERKRVSIIECLGTRASVFCWDNSTRGLD
C. albicans Cdr1p          GELTVVLGRPGAGCSTLLKTI  VSGGERKRVSIAEASLSGANIQCWDNATRGLD
S. cerevisiae Snq2p        GEMILVLGRPGAGCSSFLKVT  VSGGERKRVSIAEALAAKGSIYCWDNATRGLD
S. cerevisiae Pdr5p        GELLVVLGRPGSGCTTLLKSI  VSGGERKRVSIAEVSICGSKFQCWDNATRGLD
S. cerevisiae Ste6p        GQFTFIVGKSGSGKSTLSNLL  LSGGQQQRVAIARAFIRDTPILFLDEAVSALD
S. cerevisiae Ycf1p        GNLTCIVGKVGSGKTALLSCM  LSGGQKARLSLARAVYARADTYLLDDPLAAVD
H. sapiens MDR1            GQTVALVGNSGCGKSTTVQLM  LSGGQKQRIAIARALVRNPKILLLDEATSALD
H. sapiens CFTR            GQLLAVAGSTGAGKTSLLMMI  LSGGQRARISLARAVYKDADLYLLDSPFGYLD
                           *    . *  *.* ..       .***.. *...          *     .*

C-TERMINAL DOMAIN          Walker A          ABC signature          Walker B
                           ________          _____________          ________
A. nidulans AtrAp          GTLTALMGVSGAGKTTLLDVL  LNVEQRKLLTIGVELPPSPKLLLFLDEPTSGLD
A. nidulans AtrBp          GMLGALMGSSGAGKTTLLDVL  LSVEQRKRVTIGVELVSKPSILIFLDEPTSGLD
C. albicans Cdr1p          GQITALMGASGAGKTTLLNCL  LNVEQRKRLTIGVELVAKPKLLLFLDEPTSGLD
S. cerevisiae Snq2p        GTMTALMGESGAGKTTLLNTL  LNVEQRKKLSIGVELVAKPDLLLFLDEPTSGLD
S. cerevisiae Pdr5p        GTLTALMGASGAGKTTLLDCL  LNVEQRKRLTIGVELTAKPKLLVFLDEPTSGLD
S. cerevisiae Ste6p        GQTLGIIGESGTGKSTLVLLL  LSGGQAQRLCIARALLRKS-KILILDECTSALD
S. cerevisiae Ycf1p        NEKVGIVGRTGAGKSSLTLAL  LSVGQRQLLCLARAMLVPS-KILVLDEATAAVD
S. cerevisiae Adp1p        GQILAIMGGSGAGKTTLLDIL  ISGGEKRRVSIACELVTSP-LVLFLDEPTSGLD
H. sapiens MDR1            GQTLALVGSSGCGKSTVVQLL  LSGGQKQRIAIARALVRQP-HILLLDEATSALD
H. sapiens CFTR            GQRVGLLGRTGSGKSTLLSAF  LSHGHKQLMCLARSVLSKA-KILLLDEPSAHLD
                           .    ..* .*.**...       .     . ...   .      .. *** .. .*
```

Figure 3. Clustal alignment of conserved motifs
Alignment of the Walker A and B motifs and the ABC signature from the ATP-binding cassettes of AtrAp and AtrBp with other members of the ABC transporter superfamily. Asterisks (*) indicate identical residues, dots (.) conserved substitutions

A 5.4 kb fragment of an2, containing the region hybridizing with the *PDR5*-derived probe, was subcloned and entirely sequenced. It contains an ORF of 4278 bp interrupted by three introns ranging in size from 45 to 53 bp. The positions of these introns were confirmed by sequencing of cDNA. In the 5' flanking region adenine is present at position -3 and a 9/10 conserved Kozak motif (CCACCATG TC) spans the putative translation start. A TATA

consensus (TATATA) at position -91 and five heat-shock-like elements (5/6) identical are also present. In the 3' flanking region a typical polyadenylation signal is observed (+4614 AATAAA) 180 nucleotides downstream of the putative translation stop codon. The sequence is available in the EMBL database under accession number Z68905.

The ORF encodes a protein of 1426 amino acid residues with a predicted molecular weight of 158.9 kDa and a predicted isoeletric point of 7.04. The encoded protein contains putative N-glycosylation, phosphorylation and myristylation sites. The hydropathy profile of the encoded protein is similar to that of AtrAp (results not shown). Both hydrophilic regions contain motifs closely resembling an ABC cassette. Both cassettes display similar degeneracy relative to the Walker motifs as observed in AtrAp. The protein shows homology to Pdr5p (36% identity and 58 % similarity), Snq2p (38% identity and 60% similarity) and Cdr1p (38% identity and 60% similarity). In view of the similarity of the protein with other ABC transporters, the gene present in the 5.4 kb fragment of an2 was termed *atrB* and the encoded protein AtrBp.

Percentages of similarity and identity for AtrAp and AtrBp are 58 and 38, respectively. An alignment of the ATP-binding cassettes of AtrAp and AtrBp with other members of the ABC superfamily is presented in Figure 3.

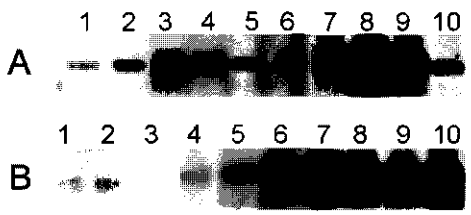

Figure 4. Northern blot analysis
Northern analysis of *atrA* (**A**) and *atrB* (**B**) using total RNA from untreated germlings of *A. nidulans* (lanes 1-5) and from germlings treated for 60 min with 10 μg ml^{-1} imazalil (lanes 6-10). Lanes 1 and 6: wild-type (strain 003), lanes 2 and 7: *imaA* mutant (strain 130), lanes 3 and 8: *imaB* mutant (strain 146), lanes 4 and 9: *imaA* + *imaB* mutant (strain R264), lanes 5 and 10: wild-type strain WG-096 used for construction of the genomic library. Equal loading of lanes with total RNA was checked by subsequent probing of the same blots with a 1.5 kb *Eco*RI+*Sal*I fragment derived from the *actin* gene of *Cladosporium fulvum*.

***atrA* and *atrB* copy number and expression in wild-type and multidrug resistant mutants**

The copy-number of *atrA* and *atrB* in the genomes of wild-type, and multidrug-resistant *ima* mutants of *A. nidulans* was studied by Southern blot analysis, using a 0.81 kb *Bam*HI-*Sst*I fragment (ID) and a 0.62 kb *Bam*HI-*Sst*I fragment (D15) (bold in Fig. 2A and 2C) as gene-

specific probes, respectively. Genomic DNA, digested with various restriction enzymes or combinations of enzymes, consistently revealed the presence of only one hybridizing fragment in all strains tested. There was no difference in hybridization intensity between strain 003 and the *ima* mutants. These results indicate that *atrA* and *atrB* are single-copy genes, which are not amplified in multidrug-resistant strains.

Transcription of *atrA* and *atrB* was investigated in wild-type and drug-resistant mutants of *A. nidulans* (Fig. 4). Northern analysis revealed the presence of a poly(A)$^+$ RNA of about 4.7 kb hybridizing with the *atrA* gene-specific probe and a slightly smaller poly(A)$^+$ RNA hybridizing with the *atrB* gene-specific probe. Both *atrA* and *atrB* are constitutively transcribed at low levels in the wild-type strain 003.

Mutants carrying the *imaB* mutation have a higher level of expression of *atrA* than both wild-type and mutant 130, carrying the *imaA* mutation (Fig. 4A). In contrast, the level of *atrB* transcript is similar in all strains analyzed (Fig. 4B).

Table 2. Ability of compounds to enhance transcription of *atrA* and *atrB* as determined by Northern analysis of total RNA extracted from 12-h-old germlings of *A. nidulans* strain 003 after treatment for 1 hour

Compound	*atrA*	*atrB*
Control	-	-
Acriflavin (3 g μml^{-1})	-	-
Cycloheximide (200 $\mu g\ ml^{-1}$)	±	++
Fenarimol (30 $\mu g\ ml^{-1}$)	-	+
Imazalil (0.3 $\mu g\ ml^{-1}$)	+	+
4-nitroquinoline oxide (1 $\mu g\ ml^{-1}$)	-	-
Pisatin (15 $\mu g\ ml^{-1}$)	-	++
Sulfomethuron methyl (30 $\mu g\ ml^{-1}$)	-	-
Tomatine (3 $\mu g\ ml^{-1}$)	-	-

-: basal level of transcripts;
±, +, + +: weak, moderate and strong increase in transcript levels, respectively

Induction of *atrA* and *atrB* transcription by drugs

Imazalil enhanced transcription levels of *atrA* in the wild-type strain 003. The effect of cycloheximide was only weak, but the antibiotic strongly increased transcription levels of *atrB*. Transcription levels of *atrB* were also enhanced by fenarimol and, more strongly, by pisatin (Table 2). An example of transcription enhancement of *atrA* and *atrB* in strain 003 by imazalil is given in Figure 4. Results in this figure also indicate that the fungicide most strongly enhanced transcription levels of *atrA* in strains 146 and R264, which carry *imaB*.

Imazalil also enhanced transcription of *atrB* but only weak differential effects among isolates were observed. A time-course experiment with strain R264 (Fig. 5) demonstrated that transcript levels of *atrA* significantly increased 10 min after treatment with imazalil, while increased transcript levels of *atrB* were already detectable 5 min after treatment with the fungicide.

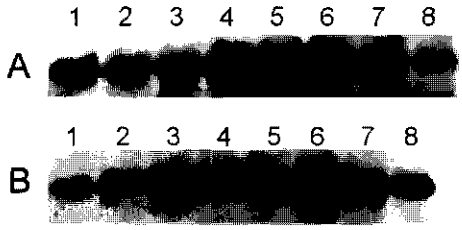

Figure 5. Northern blot analysis
Time-course of expression of *atrA* (**A**) and *atrB* (**B**) in strain R264 (*imaA* + *imaB*) of *A. nidulans* following treatment of 12-hour-old germlings with imazalil (10 μg ml^{-1}). Lanes 1 to 7: 0, 5, 10, 15, 30, 60 and 180 min of treatment respectively; lane 8: untreated control after 180 min of incubation. Equal loading of lanes with RNA was checked by subsequent probing the of the same blots with a 1.5 kb *Eco*RI+*Sal*I fragment derived from the *actin* gene of *Cladosporium fulvum*.

Functional complementation by *atrA* and *atrB* of yeast *PDR5*-null mutants

Functional analysis of *atrA* and *atrB* was performed by complementation of the *PDR5*-deficient strain JG436 of *S. cerevisiae*. To this end, full-length cDNA of *atrA* or *atrB* was cloned down-stream of the inducible promoters *GAL1* and *GAL10* in pYEura3, respectively, and transformed into the *PDR5* disruptant strain JG436. As controls, the empty vector was transformed into the parental strain RW2802 containing a functional copy of *PDR5*, and into JG436.

The sensitivity of the transformants to cycloheximide, chloramphenicol and imazalil on a galactose medium is presented in Table 3. The MIC value of cycloheximide for transformants of strain JG436 (the *PDR5*-disrupted strain) containing *atrB* and strain RW2802 (contains an intact copy of *PDR5*) is 0.3 μg ml^{-1}, which is ten times higher than the MIC value for strain JG436. The presence of *atrB* in strain JG436 also increased the MIC values for chloramphenicol and imazalil by a factor of three. Interestingly, the level of resistance to sulfomethuron methyl was even higher in transformants containing *atrB* than in strain RW2802 containing the wild-type *PDR5* gene. In contrast, MIC values of all compounds tested for transformants of JG436 containing *atrA* were similar to those of the host strain JG436. Under conditions in which the *GAL* promoter-driven expression of *atrA* and *atrB* is

repressed, i.e., in the presence of glucose, the sensitivity levels of *atrA*- and *atrB*-containing transformants did not differ from the control host strain. Furthermore, Northern analysis revealed high levels of *atrA* and *atrB* transcripts in galactose-induced transformants, while no *atrA* nor *atrB* transcripts were detected in cells grown on glucose. Taken together, these data demonstrate a causal relationship between the expression of *atrB* and the increase in drug resistance of an otherwise drug-hypersensitive, *PDR5*-deficient, yeast strain.

DISCUSSION

atrA and *atrB* are, to our knowledge, the first structural determinants of MDR reported for filamentous fungi. The putative products of both genes closely resemble other members of the ABC transporters superfamily. In particular, they share organization with the ABC transporters determining MDR in *S. cerevisiae* (Pdr5p and Snq2p) and *C. albicans* (Cdr1p). The same type of topology has been found for some "half-sized" transporters such as those encoded by the *Drosophila white* and *brown* genes, involved in the transport of eye pigments (Dreesen *et al.*, 1988; O'Hare *et al.*, 1984) and the Adp1p permease of *S. cerevisiae* (Purnelle *et al.*, 1991). In this respect, AtrAp and AtrBp differ from the P-glycoproteins encoded by the human MDR1, yeast Ste6p and many other P-glycoproteins in which the hydrophobic regions precede the hydrophilic ones.

Table 3. Effect of transformation of a *PDR5*-disrupted mutant of *S. cerevisiae* (JG436) with a full-length *atrA* (JG436 + *atrA*) or *atrB* (JG436 + *atrB*) cDNA on colony formation after 5 days of incubation in the presence of the inhibitors listed. The disrupted mutant JG436 carrying the vector plasmid (JG436 + pYEura3) served as control. RW2802 is the parental strain of JG436 with a functional copy of *PDR5*

Compound	Minimal inhibitory concentration (μg ml^{-1})			
	JG436 + pYEura3	RW2802 + pYEura3	JG436 + *atrA*	JG436 + *atrB*
Chloramphenicol	100	1000	100	300
Cycloheximide	0.03	0.3	0.03	0.3
Imazalil	10	30	10	30
Sulfomethuron methyl	1	3	1	10

Other interesting common features of the ABC transporters of *A. nidulans* and yeasts, concern the degeneration observed in the ABC cassettes as compared to those present in mammalian P-glycoproteins. The Walker A motif of mammalian P-glycoproteins contains the conserved GxSGxGK(S,T) sequence. In the Walker A motif of the N-terminal half of both

Atr proteins, the highly conserved lysine (K) is replaced by a cysteine (C) residue. This has also been described for Pdr5p and Snq2p of *S. cerevisiae* and Cdr1p of *C. albicans* (Balzi *et al.*, 1994; Prasad *et al.*, 1995; Servos *et al.*, 1993). Strikingly, substitution of K by other amino acids severely impairs transport activity of mammalian P-glycoproteins and Ste6p (Berkower and Michaelis, 1991) but not ATP binding (Azzaria *et al.*, 1989). Obviously, this is not necessarily the case for all ABC transporters of filamentous fungi and yeasts. In the Walker B motif in the N-terminal half of both Atrs, the highly conserved glutamic acid residue (E) is substituted by asparagine (N), as also found in Pdr5p, Snq2p and Cdr1p. Similar features hold for the C-terminal ABC signature in which the conserved consensus LSGGQ of mammalian P-glycoproteins is replaced by LNVEQ in AtrAp and by LSVEQ in AtrBp. The substitution in this motif of the conserved serine (S) by N in the CFTR gene product (S549N) is associated with cystic fibrosis in humans (Cutting *et al.*, 1990). In conclusion, AtrAp and AtrBp are new members of a new subfamily of ABC transporters which up to now has been observed only in yeasts.

Previous studies (De Waard and Van Nistelrooy, 1979; 1980; 1987) demonstrated that accumulation of the azole fungicide fenarimol by the wild-type strain 003 of *A. nidulans*, follows a transient pattern with a maximum at 10 min after addition of the fungicide to mycelium. Similar results have been described for other azole fungicides in *Penicillium italicum* (De Waard and Van Nistelrooy, 1984; 1988) and *Nectria haematococca* (Kalamarakis *et al.*, 1991). Isogenic mutants of *A. nidulans* strain 003 carrying *imaA* and *imaB* mutations, displayed a relatively low and constant level of fungicide accumulation over time. It was proposed that the transient accumulation of azoles by the wild-type *A. nidulans* is based on the capacity of azole fungicides to induce an energy-dependent efflux activity responsible for active extrusion of the compounds, while efflux activity in the mutants was referred to be constitutive (De Waard and Van Nistelrooy, 1981). The rapid increase in expression of *atrA* and, especially, *atrB* upon treatment with azole fungicides coincides with induction of azole efflux from mycelium in the wild-type strain, suggesting that both Atrs can play a role in extrusion of the fungicides.

imaB mutants (146, R264) have a higher basal level of expression of *atrA* than the wild-type strain. The mutants also showed the strongest activation of *atrA* transcription after treatment with imazalil. This relationship between the presence of *imaB* and expression of *atrA* indicates that the *imaB* locus regulates *atrA* expression, perhaps acting as a regulatory protein with a function similar to the one exerted by Pdr1p on *PDR5* in *S. cerevisiae*. The Pdr1p regulatory protein is able to influence the expression of several genes, including *PDR5*

(Balzi and Goffeau, 1995). Further studies will aim to establish such a relationship between *imaB* and *atrA*.

The basal level of *atrB* expression was similar in all strains tested. In all strains, imazalil strongly activated transcription to about the same extent. Similar results were found for cycloheximide and pisatin (results not presented). Hence, no correlation was observed between the presence of *imaA* or *imaB* mutations and the expression pattern of *atrB*. Therefore, *atrB* may be subject to stress-induced transcriptional activation as described for *PDR5* in *S. cerevisiae* (Miyahara *et al.*, 1996), characterized by an even faster inducibility than *atrA*.

Compounds that induce the expression of atr genes are not always involved in the pleiotropic effects observed for the *imaA* and *imaB* mutants. For instance, the basal level of expression of *atrA* is higher in *imaB* carrying strains and the enhancement in transcript levels caused by cycloheximide treatment makes this difference even larger. Nevertheless, no significant difference in cycloheximide resistance is observed between wild-type and *imaB* mutants. This implies that, at least for some compounds, an increase in *atr* transcription is not sufficient to confer drug resistance. In the case of cycloheximide, AtrAp synthesis may be inhibited at the translational level. Similar results were obtained in *S. cerevisiae* in which expression of the multidrug transporters *YDR1* (=*PDR5*), and *SNQ2* can also be induced by compounds that are not relevant to multidrug resistance (Hirata *et al.*, 1994).

The functional role of *atrA* and *atrB* in multidrug resistance has been analyzed by complementation of a *PDR5*-deficient yeast mutant. Transformants containing *atrB* displayed an increase in resistance to several drugs. Interestingly, the spectrum of resistance conferred by *atrB* is different from the one displayed by a yeast strain containing an intact copy of *PDR5*. This indicates that AtrBp is functionally different from Pdr5p, implying that ABC transporters may have partially overlapping substrate specificities (e.g. cycloheximide), as it was found for Pdr5p and Snq2p. Studies with mammalian P-glycoproteins demonstrated that drug specificity can be determined by the amino acid sequence in specific regions. In human MDR cell lines, two separate nucleotide substitutions in the sixth N-terminal transmembrane domain completely changed the pattern of resistance (Devine *et al.*, 1992) and even single amino acid substitution in a mouse *mdr* gene product can abolish its ability to complement a yeast *STE6* null mutant (Raymond *et al.*, 1992). Mutational analysis of *atrA* and *atrB* will be the object of future studies.

Additional insight into the role of both *atrs* in multidrug resistance will be obtained by studying the drug resistance spectra in *A. nidulans* disruption mutants. This approach may

also be useful in determining the function of *atrA*, which failed to complement the *PDR5* deficiency in yeast, although high levels of *atrA* transcripts were detected in JG436 cells transformed with the *atrA* cDNA (results not shown). It is possible that *atrA* mRNA is incorrectly processed and/or sorted in yeast, thus giving a non-functional product. Disturbance of directional transport might also be one reason why AtrAp did not complement Pdr5p. Alternatively, AtrAp could also mediate secretion of endogenous compounds (i.e., secondary metabolites, pheromones, etc.) or specifically be involved in extrusion of drugs different from those substrates of Pdr5p. The latter hypothesis is supported by data presented in Table 2.

Based on the results described, we hypothesize that the ABC transporters encoded by *atrA* and *atrB* play a role in protecting the saprophytic fungus *A. nidulans* from natural toxic compounds. A similar function was proposed for P-glycoproteins of the nematode *Caenorhabditis elegans* (Broeks *et al.*, 1995). Support for this hypothesis comes from the MDR pattern, expression and complementation studies. This hypothesis not only relates to antibiotics but also to plant defense products such as the phytoalexin pisatin. Hence, it corroborates the findings that pisatin-induced transcription of *atrB* and tolerance of *N. haematococca* to pisatin can be mediated by an energy-dependent efflux (Denny *et al.*, 1987; Denny and VanEtten, 1983). If ABC transporters enable plant pathogens to extrude plant defense products, thereby reducing their accumulation levels in fungal cells, they might constitute a novel class of pathogenicity factors. This hypothesis is being tested by studying ABC-transporter-encoding genes in the plant pathogen *Botrytis cinerea* and the role of such genes in pathogenicity. One *PDR5* homologue of *Botrytis cinerea* has already been cloned and characterized (Del Sorbo and De Waard, 1996).

Finally, we also propose that *atrA* and, especially, *atrB* are ideal models for the study of resistance to therapeutic drugs and agricultural fungicides in mammalian and plant pathogens.

EXPERIMENTAL PROCEDURES

Chemicals

Fenarimol, imazalil, triadimenol and sulfomethuron methyl were kindly provided by Dow Elanco (Greenfield, Ind., USA), Janssen Pharmaceuticals (Beerse, Belgium), Bayer AG (Leverkusen, Germany), and DuPont De Nemours (Wilmington, Del. USA), respectively. Pisatin was purified from pea pods (Fuchs *et al.*, 1981). All other chemicals were from Sigma (St. Louis, Mo.) unless otherwise indicated.

Strains, plasmids and toxicity tests

Strains of *A. nidulans* used in this study were the wild-type 003 (*biA1*; *acrA1*) and imazalil-resistant isogenic mutants carrying *imaA* (130), *imaB* (146) or both mutations (R264) (Van Tuyl, 1977). Sensitivity to toxicants was determined by measuring their EC_{50} values for inhibition of radial growth or the minimal inhibitory concentrations (MIC) necessary to prevent colony formation (De Waard and Van Nistelrooy, 1979). Compounds tested included antibiotics (cycloheximide, neomycin, oligomycin, valinomycin), plant defense products (pisatin and tomatine), azole and related fungicides which inhibit sterol 14α-demethylation (imazalil, fenarimol and triadimenol), a herbicide (sulfomethuron methyl), calmodulin antagonists (chlorpromazine and trifluprομazine), and other xenobiotics (acriflavin and 4-nitroquinoline-oxide). A strain was considered resistant or hypersensitive when the EC_{50} value of a particular compound was at least two times higher or lower than the EC_{50} value of the wild-type strain, respectively. The *S. cerevisiae PDR5* null mutant JG436 (MAT**a**, *PDR5*::*Tn5*, *leu2*, *met5*, *ura3-52*, *mak71*, *KRB1*; Meyers *et al.*, 1992) and yeast plasmid pYEura3 were kindly provided by Professor A. Goffeau.

Library screening, Southern and Northern hybridizations

A genomic library of *A. nidulans* strain WG-096 in EMBL3 was kindly provided by Dr. T. Goosen (Department of Genetics, Wageningen Agricultural University, Wageningen, The Netherlands). Positive phage clones were selected using a 1.52 kb *Bgl*II fragment starting 0.37 kb before the translation start codon and comprising the entire N-terminal ATP-binding cassette (ABC) of gene *PDR5* from *S. cerevisiae*.

DNA and RNA of *A. nidulans* were extracted according to Raeder and Broda (1985) and Longmann et al. (1987), respectively, from 12-hour-old liquid cultures shaken at 37 °C after inoculation with 10^7 conidia ml^{-1}. Southern and Northern hybridizations were performed on HybondN^+ and HybondN nylon membranes (Amersham), respectively, according to the manufacturer's instructions. Blots were washed at 65 °C in 0.1X SSC unless otherwise indicated. In heterologous hybridizations, blots were washed at 56 °C using 1X SSC.

Other recombinant DNA manipulations

DNA fragments from phages that were positive after tertiary screening were cloned in pGEM-3Zf(+) (Promega, Madison, Wis.) or pBluescript IISK vectors (Stratagene, La Jolla, Calif.). *E.coli* DH5α was used as bacterial host for propagation of constructs. Sequencing was carried out by the dideoxy-chain-termination method (Sanger *et al.*, 1977), using Taq polymerase and

fluorescent dye terminator dideoxynucleotides. Intron-exon junctions were confirmed by sequencing a cDNA cloned in pGEM-T. Sequences were analyzed with the GCG Package software (Genetics Computer Group, Madison, Wis.). Multiple alignment was done using the CLUSTAL program.

First-strand cDNA of *atrA* and *atrB* was synthesized on poly(A)$^+$ RNA of germlings from *A. nidulans* strain 003, using the Superscript TM II kit (Gibco-BRL, Garthersburg, Md.) according to manufacturer's instructions. PCR amplification of cDNA was performed with the Expand High Fidelity kit (Boehringer, Mannhein GmbH, Mannheim, Germany) using a Techne PHC3 thermocycler (New Brunswick Scientific, Nijmegen, The Netherlands). Primers used in PCR reactions were provided by Pharmacia Biotech Benelux (Roosendaal, The Netherlands). Primers for amplification of cDNA were designed across the putative translation start (5'-GTTCATTCTAGACACCATGGGTGTTCC-3') and stop codon on (5'-ATGCTCTAGACATCTCACTTCTTCC-3') of the *atrA* gene. Artificial *Xba*I sites were included to allow subcloning of the PCR product. For the same purpose, *Eco*RI sites were included in the primers for amplification of the cDNA of *atrB* (primer sequences were 5'-TTCGAATTCCATGTCTACCCTCACCGTG-3' and 5'-TTCGAATTCGTAAAAGCCTACTCCTCTGC-3', respectively). Amplified PCR products were cloned in pGEM-T (Promega) and propagated in *E. coli*.

For yeast complementation studies, *atrA* and *atrB* cDNAs were cloned into the *Xba*I or *Eco*RI site of the yeast shuttle vector pYEura3, under the control of galactose-inducible promoters *GAL1* and *GAL10*, respectively. The correct orientation of the coding regions was checked by restriction analysis. Transformation of *S. cerevisiae* strain JG436 was performed by the electroporation method described by Becker and Guarante (1991). The resistance of Ura$^+$ transformants to a series of compounds was tested on a synthetic medium (Balzi *et al.*, 1987) containing raffinose and galactose as carbon sources, inoculated with small droplets of standardized cell suspensions, according to Prasad et al. (1995).

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Chapter 3

The role of ABC transporters from *Aspergillus nidulans* in protection against cytotoxic agents and antibiotic production

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SUMMARY

This paper describes the characterization of *atrC* and *atrD* (*A*BC *tr*ansporters C and D), two novel ABC-transporter encoding genes from the filamentous fungus *Aspergillus nidulans*, and provides evidence for the involvement of *atrD* in multidrug transport and antibiotic production. BLAST analysis of the deduced amino acid sequences of AtrCp and AtrDp reveals high homology to ABC-transporter proteins of the P-glycoprotein cluster. AtrDp shows a particularly high degree of identity to the amino acid sequence of AfuMdr1p, a previously characterized ABC transporter from the human pathogen *Aspergillus fumigatus*. Northern blot analysis demonstrates an increase in transcript levels of *atrC* and *atrD* in fungal germlings upon treatment with natural toxic compounds and xenobiotics. The *atrC* gene has a high constitutive level of expression relative to *atrD*, which suggests its involvement in a metabolic function. Single knock-out mutants for *atrC* and *atrD* were generated by gene replacement using the *pyrG* from *Aspergillus oryzae* as a selectable marker. Δ*atrD* mutants display a hypersensitive phenotype to compounds such as cycloheximide, the cyclosporin derivative PSC 833, nigericin and valinomycin, indicating that AtrDp is involved in protection against cytotoxic compounds. Energy-dependent efflux of the azole-related fungicide fenarimol is inhibited by substrates of AtrDp (*e.g.* PSC 833, nigericin and valinomycin), suggesting that AtrDp plays a role in efflux of this fungicide. Most interestingly, Δ*atrD* mutants display a decrease in penicillin production measured indirectly as antimicrobial activity against *Micrococcus luteus*. These results suggest that ABC transporters may be involved in secretion of penicillin from fungal cells.

INTRODUCTION

ATP-Binding Cassette (ABC) transporters are highly conserved traffic ATPases that occur ubiquitously in nature (Higgins, 1992). Some members of this large superfamily of proteins function in transport of cytotoxic agents across biological membranes, resulting in reduced intracellular accumulation of toxins. Hence, they play a role in protecting cells against natural toxins. ABC transporters have become especially known for their role in multidrug resistance (MDR) in human tumour cells. The MDR family of transporters includes the multidrug resistance P-glycoprotein (P-gp) encoded by the *MDR1* gene in

humans, and the human multidrug resistance-associated protein MRP1 (MRP); both are plasma membrane proteins which catalyze the ATP-dependent extrusion of anti-tumour drugs during chemotherapy of cancer cells (Cole *et al.*, 1992; Gottesman and Pastan, 1993). The major drug efflux pumps identified in microorganisms belong to the Major Facilator (MF) and the ABC transporter superfamilies of proteins (Marger and Saier, 1993; Van Veen and Konings, 1998). Analysis of the complete genome sequence of *Saccharomyces cerevisiae* revealed 29 ABC transporter-encoding genes, and overproduction of at least four of them results in MDR (Decottignies and Goffeau, 1997). In other yeast species such as *Schizosaccharomyces pombe* and the human pathogen *Candida albicans*, MDR based on overproduction of ABC transporters has also been described. Examples are Cdr1p and Cdr2p from *C. albicans*, and Pmd1p and Bfr1p from *S. pombe* (Nagao *et al.*, 1995; Nishi *et al.*, 1992; Prasad *et al.*, 1995; Sanglard *et al.*, 1996, 1997).

MDR in filamentous fungi was first reported for mutants of *Aspergillus nidulans* generated in the laboratory and selected for resistance to azole fungicides (Van Tuyl, 1977). Resistance of these mutants to azoles and related fungicides was based on an energy-dependent efflux mechanism, which results in decreased accumulation of the drug in the cytoplasm (De Waard and Van Nistelrooy, 1979). The isolation and characterisation of two ABC-transporter encoding genes (*atrA* and *atrB*) from this fungus have been reported (Del Sorbo *et al.*, 1997). Both genes encode proteins that display a high degree of homology to Pdr5p, an ABC transporter from *S. cerevisiae* involved in MDR (Balzi *et al.*, 1994; Bissinger and Kuchler, 1994; Hirata *et al.*, 1994). In field isolates of the phytopathogenic fungus *Penicillium digitatum*, the causal agent of citrus green mold, the ABC transporter Pmr1p plays a role in azole resistance (Nakaune *et al.*, 1998). Protection against a phytoalexin from rice has been postulated as the function of Abc1p, an ABC transporter from the rice pathogen *Magnaporthe grisea* that is essential for pathogenicity (Urban *et al.*, 1999). The ABC transporter genes *AfuMDR1* and *AfuMDR2*, and *AflMDR1* have been described for *Aspergillus fumigatus* and *Aspergillus flavus*, respectively. AfuMdr1p may be involved in drug transport since it confers decreased sensitivity to the antifungal compound cilofungin when overexpressed in yeast (Tobin *et al.*, 1997).

Endogenous substrates of ABC transporters involved in MDR are largely unknown, but phospholipids have been suggested as candidates (Decottignies *et al.*, 1998; Kamp and Haest, 1998; Mahe *et al.*, 1996; Van Helvoort *et al.*, 1996). It has been suggested that fungal ABC transporters can also be involved in transport of secondary

(toxic) metabolites (De Waard, 1997). *A. nidulans* is a well known producer of various toxic secondary metabolites, such as sterigmatocystin and penicillin. The biosynthetic pathway for these compounds has been characterized at the molecular level but little is known about the transport of these compounds and their precursors over biological membranes (Brakhage, 1998; Brown *et al.*, 1999; Penalva *et al.*, 1998).

In this paper we report on the isolation and functional characterisation of *atrC* and *atrD*, two novel ABC transporter-encoding genes from the filamentous fungus *A. nidulans*, and provide evidence suggesting that AtrDp is a novel multidrug transporter protein that plays a role in antibiotic secretion.

RESULTS

The primary amino acid sequences of AtrCp and AtrDp are highly homologous to those of MDR proteins

A PCR-based approach using degenerate primers designed from conserved domains of ABC transporters involved in MDR from a variety of organisms resulted in the amplification of two DNA fragments from *A. nidulans*. Cloning and DNA sequence analysis revealed that the amplified fragments were different and encoded highly conserved amino acid sequences, characteristic of proteins containing an ATP-binding cassette (Bairoch, 1992; Walker *et al.*, 1982). The fragments were used as probes to screen a genomic library of *A. nidulans*. For each probe, positive lambda clones were isolated and purified. Southern analysis confirmed the presence of identical hybridizing restriction fragments in genomic DNA and in the positive lambda clones isolated. Overlapping subclones from phage inserts were cloned and sequenced. The sequence of a 6120-bp contig from one lambda clone revealed the presence of an open reading frame (ORF) of 3852 bp, interrupted by four introns, ranging in size from 46 to 65 bp. The positions of the introns were confirmed by cDNA sequencing (Fig. 1A). Analysis of the deduced 1284-amino acid sequence of the encoded protein, named AtrCp (**A**BC **tr**ansporter **C**), suggested the presence of 12 transmembrane (TM) domains and two nucleotide binding domains (NBD). These are arranged in two homologous halves in a $(TM_6\text{-}NBD)_2$ configuration, as predicted by the TMpred software (Hofmann and Stoffel, 1993). The DNA sequence of the second PCR fragment was not present in *atrC*. Screening of the phage library with this fragment resulted in another ABC transporter gene, designated *atrD*. Sequence analysis of *atrD* revealed the presence of an ORF interrupted by two confirmed introns, encoding a putative protein of 1348 amino acids with the same

topology as AtrCp (Fig. 1B). BLAST analysis of the deduced amino acid sequence of AtrCp and AtrDp revealed strong homology to ABC transporters, in particular to the P-glycoprotein sub-family (Table 1). Alignment of the conserved motifs of AtrCp and AtrDp indicates a high degree of homology with other ABC transporters (Fig. 2). The degree of homology between AtrDp and AfuMdr1p is remarkably high, with overall identity of 76% (Table 1), while the N and C-terminal NBDs are almost identical (Fig. 2). The best characterized ATP-binding subunit of ABC transporters has been described for the HisP protein from *Salmonella typhimurium*. Residues of HisP depicted in bold (Fig. 2) represent amino acids that interact with ATP (Hung *et al.*, 1998) and are highly conserved in other ABC transporters.

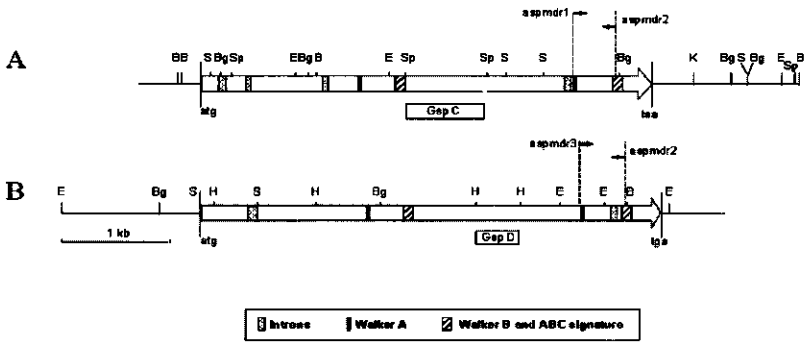

Figure 1. Physical map of the genomic regions encoding *atrC* (A) and *atrD* (B)
The open arrows represents the coding region of the *atr* genes interrupted by introns. The conserved motifs characteristic of ABC-transporter proteins are shown. The PCR fragments amplified using degenerate oligonucleotide primers (*aspmdr1*, *aspmdr2* and *aspmdr3*) are located between the dashed vertical lines. The boxes labeled GspC and GspD represent the DNA fragments used as gene-specific probes. Restriction sites are abbreviated as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I and Sp, *Sph*I.

Table 1. Pairwise comparison of deduced amino acid sequences of *atrC* and *atrD* from *Aspergillus nidulans* and other ABC transporters classified in the P-glycoprotein subfamily.

Protein	Organism	GenBank number	AtrCp			AtrDp		
			Blast score [a]	Identity (%)	Similarity (%)	Blast score	Identity (%)	Similarity (%)
AtrCp	*A. nidulans*	AF071410	-	-	-	0	36	53
AtrDp	*A. nidulans*	AF071411	0	36	53	-	-	-
AfuMdr1p	*A. fumigatus*	U62933	0	37	54	0	76	83
AflMdr1p	*A. flavus*	U62931	0	35	52	0	57	71
Pmd1p	*S. pombe*	P36619	0	36	51	0	45	60
CneMdr1p	*C. neoformans*	U62930	0	34	51	0	42	57
ChMdr1p	*C. griseus*	P21448	0	33	51	0	38	55
HsMDR1	*H. sapiens*	P08183	0	32	50	0	39	55
HvMdr2p	*H. vulgare*	Y10099	e^{-158}	31	48	0	34	51
Ste6p	*S. cerevisiae*	P12866	e^{-84}	24	43	e^{-113}	24	43

[a] Results were obtained with the BLASTp program of sequence alignment (Altschul *et al.*, 1997).

Southern analysis of genomic DNA of *A. nidulans* digested with different restriction enzymes using the gene-specific probes GspC and GspD demonstrate that *atrC* and *atrD* are single-copy genes (results not shown). A schematic representation of GspC and GspD is presented in Fig. 1A and 1B, respectively.

Figure 2. Alignment. Amino acid sequence alignment of the conserved NBD motifs of AtrCp and AtrDp with those of other ABC transport proteins. Sequences were aligned using the CLUSTAL W program (Thompson *et al.*, 1994). The asterisks indicate identical residues and dots indicate conservative substitutions. The conserved motifs (A) Walker A, (B) Walker B, (C) ABC signature and (D) the highly conserved histidine residue as detected by Decottignies and Goffeau (1997) are highlighted against a gray background. Residues in bold indicates residues that interact with ATP (Hung *et al.*, 1998).

Transcription of *atrC* and *atrD* is enhanced by xenobiotics

To verify the possible involvement of AtrCp and AtrDp in drug transport, we investigated the level of transcription of these genes upon treatment of *A. nidulans* germlings with various toxicants. Results show that the basal level of *atrC* expression is higher than that of *atrD* (Fig. 3). The plant secondary metabolites reserpine and pisatin, the azole fungicide imazalil and the protein synthesis inhibitor cycloheximide, enhance transcription of both *atr* genes, while the azole-related fungicide fenarimol specifically enhances transcription of *atrD* (Fig. 3).

* The sequence of *AnatrC* (AF071410) was submitted to the GenBank database (confidential) by June 9, 1998. Later an additional ABC transporter gene from *A. nidulans* was filed under the same name of *atrC*, on August 4, 1998. Part of the sequence of this gene is published without reference to its function (Angermayr *et al.*, 1999). We propose to rename the latter gene as *AnatrC2*.

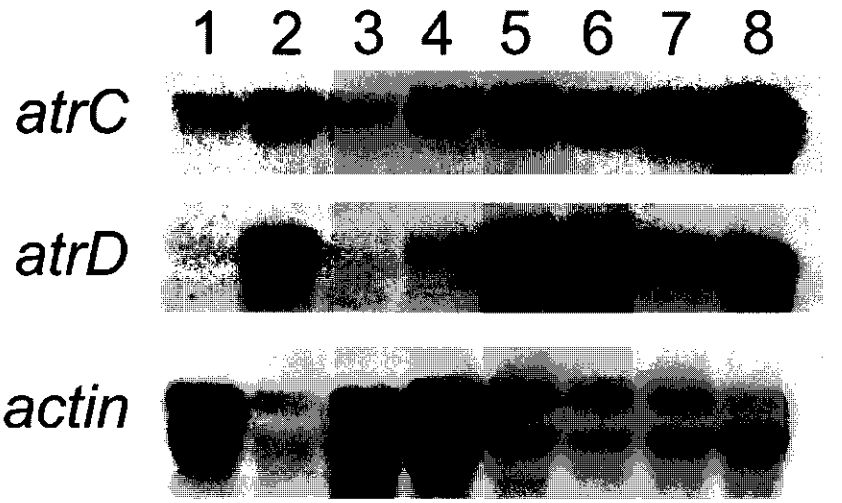

Figure 3. Northern blot analysis
Northern analysis of *atrC* (top) and *atrD* (middle) using total RNA from germlings of *Aspergillus nidulans* treated with toxicants for 60 min. Lane 1, control (0.1% DMSO); 2, reserpine (100 μg ml^{-1}); 3, sulfomethuron methyl (30 μg ml^{-1}); 4, control (0.1% ethanol); 5, imazalil (10 μg ml^{-1}); 6, fenarimol (20 μg ml^{-1}); 7, pisatin (15 μg ml^{-1}); 8, cycloheximide (20 μg ml^{-1}). A radiolabeled fragment of the actin encoding gene from *A. nidulans* was used as loading control (bottom).

Δ*atrD* strains are hypersensitive to known substrates of MDR proteins

In order to functionally characterize *atrC* and *atrD*, deletion alleles for each gene were generated by gene replacement. The major part of the coding region of the *atr* genes was replaced by the orotidine-5'-phosphate decarboxylase (*pyrG*) encoding-gene of *A. oryzae*, using an uridine-auxotrophic mutant (WG488) of *A. nidulans* as the recipient strain for transformation. Selection of transformants was based on uridine prototrophy. The use of the heterologous selectable marker of *A. oryzae* minimizes the chance that the construct will integrate at the *pyrG* locus of *A. nidulans*. A schematic representation of the disruption strategy used is given in Fig. 4A and B. A pre-selection step was performed among 24 transformants per *atr* gene by dot-blot analysis. Blots containing spotted genomic DNA of transformants were hybridized with the gene-specific probes GspC and GspD of *atrC* and *atrD*, respectively. Southern analysis of 8 pre-selected transformants per *atr* gene confirmed single-copy replacement of both genes. The frequency of single-copy replacements was 16% for *atrC* and 8% for *atrD*. Further Southern analysis of two independent transformants of *atrC* (probe C1) and *atrD* (probe D1) was performed and

confirmed replacement of the wild-type locus (Fig. 4C and D). Northern blot analysis was carried out with total RNA isolated from germlings treated with cycloheximide, a strong inducer of *atrC* and *atrD* transcription. This treatment did not reveal any transcripts from *atrC* and *atrD* in the Δ*atrC* and Δ*atrD* strains, respectively (Fig. 4E). These observations confirm that *atrC* and *atrD* were functionally deleted.

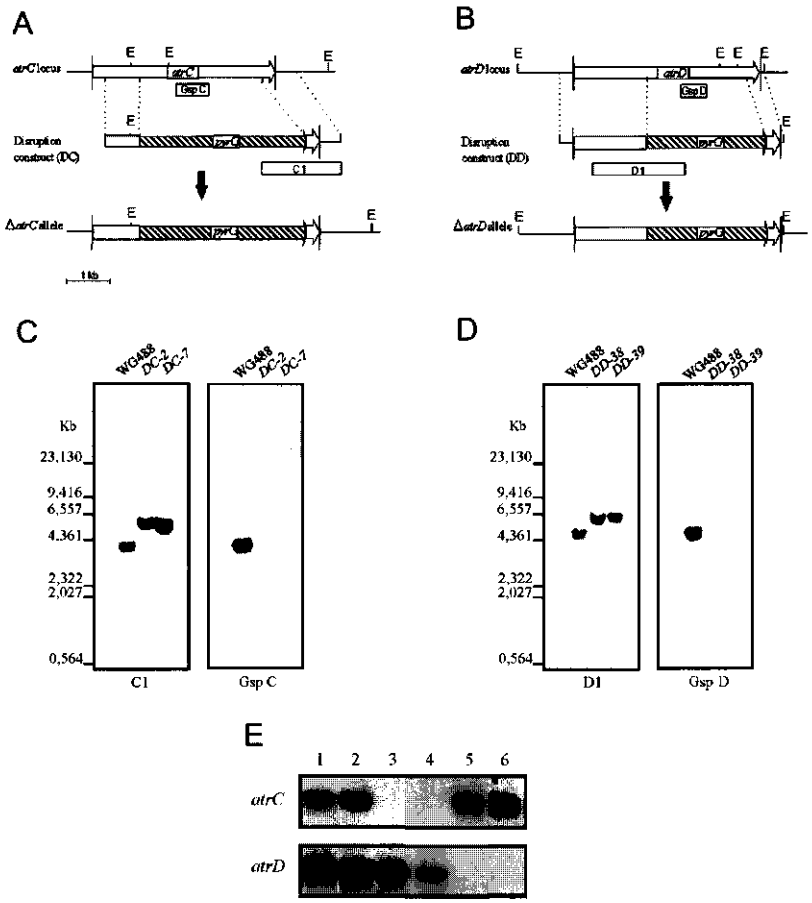

Figure 4. Replacement of the *Aspergillus nidulans atrC* and *atrD* genes.
A, B. Schematic representation of the disruption construct, and wild-type and disrupted locus of *atrC* **(A)** and *atrD* **(B)**. The open boxes labelled GspC, GspD, C1 and D1 indicate the restriction fragments used as probes in Southern and Northern analyses. Southern analysis was performed with the recipient strain WG488 used for transformation and two independent monospore isolates of the disruptants: DC-2 and DC-7 for disruptants of *atrC* and DD-38 and DD-39 for disruptants of *atrD*. **C.** Genomic DNA of WG488, DC-2 and DC-7 was restricted with *Eco*RI and hybridized with probes C1 and GspC. **D.** Similarly, a blot containing *Eco*RI-restricted genomic DNA of strains WG488, DD-38 and DD-39 was hybridized with the D1 and GspD probes. **E.** Northern analysis of germlings of *A. nidulans* treated with cycloheximide (20 μg ml^{-1}) for 60 min. The lanes were loaded with RNA from the control strains PAO-1 (1) and PAO-2 (2) transformed with the pAO-2 vector containing the *pyrG* gene of *A. oryzae*, the Δ*atrC* strains DC-2 (3) and DC-7 (4) and the Δ*atrD* strains DD-38 (5) and DD-39 (6). The upper panel shows the result of a hybridization with the gene-specific probe GspC of *atrC* and the lower panel shows the same blot hybridized (after stripping the first probe) with the gene-specific probe GspD of *atrD*.

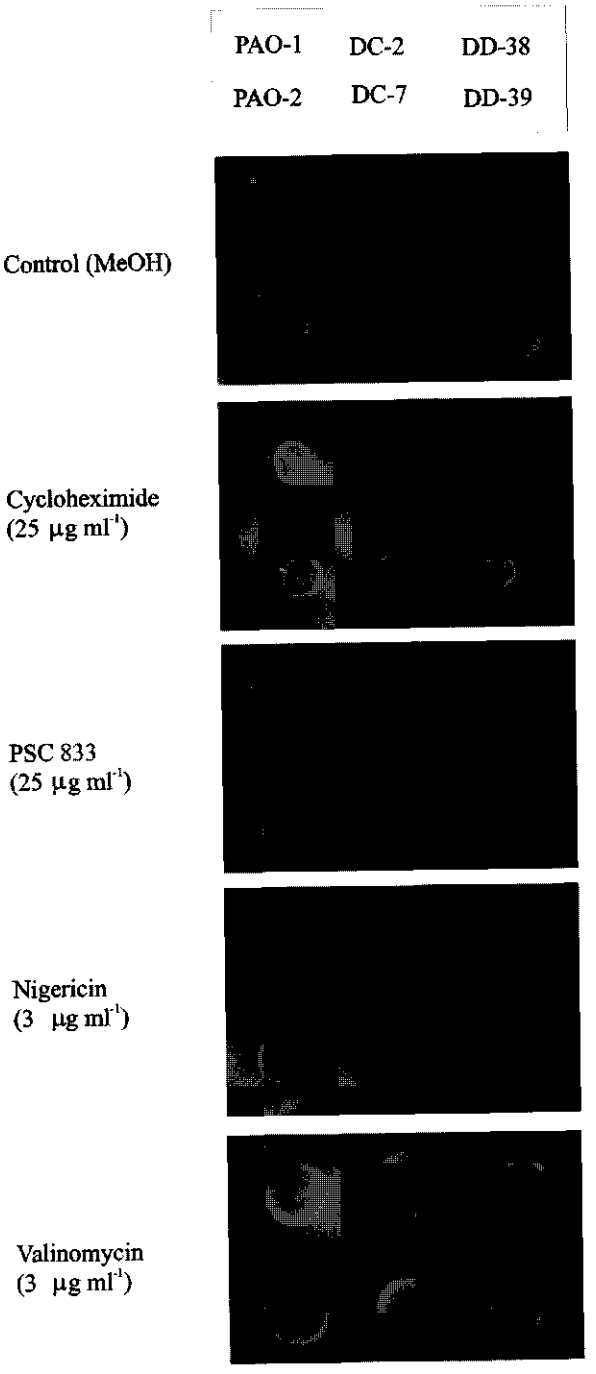

Figure 5. Toxicity assays
Sensitivity of *Aspergilus nidulans* strains PAO-1 and PAO-2 (controls), DC-2 and DC-7 (Δ*atrC*) and DD-38 and DD-39 (Δ*atrD*) to four structurally unrelated compounds. Mycelial agar plugs of a confluent plate (incubated overnight) of each strain were placed upside down on a minimal medium (MM) plate containing the indicated concentration of the compound. Radial growth was assessed after 3 days incubation, at 37 ^{0}C.

Two independent monospore isolates of *ΔatrC* (DC-2 and DC-7) and *ΔatrD* (DD-38 and DD-39) were characterized. The deletion mutants grow normally and no differences in radial growth rates were observed. A radial growth toxicity test was used to evaluate the role of AtrCp and AtrDp proteins in drug transport. Activity of thirty five compounds (see experimental procedures) was tested. None of them differentially inhibited growth of *ΔatrC* monospore isolates and control isolates PAO-1 and PAO-2. In contrast, *ΔatrD* mutants displayed increased sensitivity to cycloheximide (25 μg ml^{-1}), the cyclosporin derivative PSC 833 (25 μg ml^{-1}), nigericin (3 μg ml^{-1}), and valinomycin (3 μg ml^{-1}) as compared to the control isolates tested (Fig. 5).

The role of AtrDp in the energy-dependent efflux of [^{14}C]fenarimol

MDR in *A. nidulans* was first reported for laboratory-generated mutants selected for resistance to azole fungicides and related compounds (Van Tuyl, 1977). In these genetically defined MDR mutants, resistance to fenarimol is based on increased energy-dependent efflux activity that results in decreased drug accumulation in the cytoplasm (De Waard and Van Nistelrooy, 1979, 1980). This efflux-activity is sensitive to vanadate. In order to assess the role of AtrDp in this efflux-mechanism, we tested the potency of identified substrates and transcriptional inducers of *atrD* to inhibit the efflux of [^{14}C]fenarimol. Pronounced inhibitory effects were observed for the cyclosporin derivative PSC 833, nigericin, reserpine and valinomycin (Fig. 6A). Interestingly, the effect of reserpine is transient while that of nigericin, valinomycin and the cyclosporin derivative PSC 833 is proportional to the time of exposure to the test compound. Cycloheximide has no pronounced effect on [^{14}C]fenarimol accumulation, when applied 60 min after addition of the labeled fungicide. However, when applied 60 min prior to addition of the fungicide, inhibition of efflux activity was observed (data not shown). The parent strain PAO-2 and the deletion mutants of *atrC* and *atrD* (DC-7 and DD-39, respectively) display a similar transient accumulation of [^{14}C]fenarimol (Fig. 6B).

***ΔatrD* mutants show decreased secretion of antibiotic activity**

To test the hypothesis that ABC transporters can export endogenous secondary metabolites, antibiotic activity secreted from the disruptants was studied in a bioassay using *Micrococcus luteus* as a test organism. Antibiotic activity of *ΔatrD* strains proved to be significantly lower (33 % reduction) than that of control and *ΔatrC* strains (Fig.

7A and 7C). Inhibition zones in the bioassay disappeared when the agar was supplemented with the enzyme penicillinase (Fig. 7B), indicating that the antibiotic activity was due to the production of penicillin or related antibiotics.

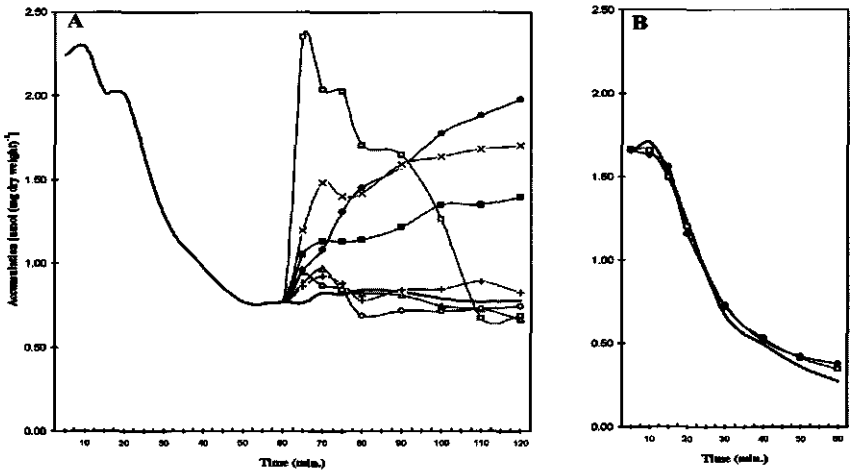

Figure 6. Accumulation of [^{14}C]fenarimol by germlings of *Aspergillus nidulans*
A. Effect of cycloheximide (Δ), PSC 833 (■), nigericin (●), valinomycin (×), quercetin (○), and reserpine (□) on [^{14}C]fenarimol accumulation by germlings of strain PAO-2. Chemicals (at 100 μg ml^{-1}) were added 60 min after addition of [^{14}C]fenarimol (t=0). Controls: methanol (0.1 %, bold line), DMSO (0.1%, +). **B.** Comparison of accumulation by germlings of strains PAO-2 (bold line), DC-7 (□) and DD-39 (●). [^{14}C]fenarimol was added to germlings at t=0.

DISCUSSION

We have cloned and functionally characterized two novel ABC transporter-encoding genes, named *atrC* and *atrD*, from the filamentous fungus *A. nidulans*. The encoded proteins are highly homologous to previously characterized ABC-transporter proteins from the human pathogens *A. flavus* (AflMdr1p) and *A. fumigatus* (AfuMdr1p), as well as to the leptomycin B resistance protein Pmd1p from *S. pombe* (Nishi *et al.*, 1992; Tobin *et al.*, 1997). AtrDp is 76% identical to AfuMdr1p. The number and position of introns in *atrD* and *Afu*Mdr1p are conserved. These results suggest a close evolutionary relationship between these two proteins. AfuMdr1p confers decreased sensitivity to the antifungal agent cilofungin when overexpressed in *S. cerevisiae* (Tobin *et al.*, 1997). However, *ΔatrD* strains of *A. nidulans* displayed wild-type sensitivity to that compound. Hence, the high degree of primary sequence homology does not imply a similar substrate specificity.

The basis of substrate specificity of ABC proteins is largely unknown. ABC transporters involved in MDR from various organisms can share a similar set of substrates

but can vary significantly in primary sequence, topology and size. For instance, the Pdr5p protein from *S. cerevisiae* and the human MDR1 P-glycoprotein share substrate specificity, despite a difference in topology and low sequence homology (Kolaczkowski *et al.*, 1996).

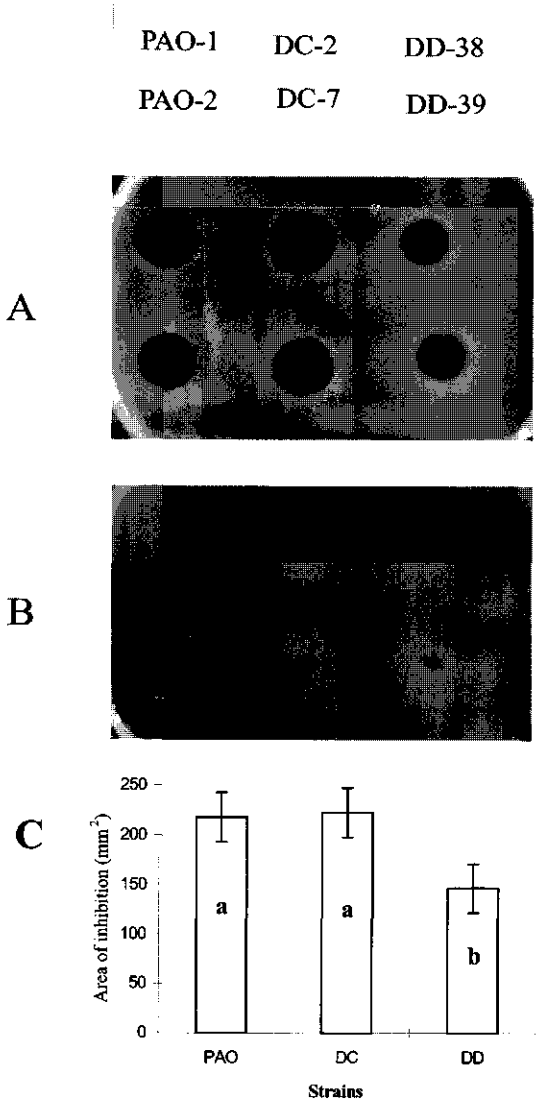

Figure 7. Secreted antibiotic activity
Agar plugs taken from the center of 14-day-old colonies of *Aspergillus nidulans*, grown on complete medium (CM) plates at 25 ^{0}C, were placed on agar plates seeded with *Micrococcus luteus* and incubated overnight at 37 ^{0}C. **A.** Inhibition zones indicating antibacterial activity of control strains PAO-1 and PAO-2, Δ*atrC* strains DC-2 and DC-7, and Δ*atrD* strains DD-38 and DD-39. **B.** A replicate of plate A amended with penicillinase (10 units). **C.** The bars represent the means of the area of inhibition (mm^2) obtained from 6 replicates. Analysis of variance and comparisons between means were applied as described by Snedecor and Cochran (1989). Identical letters within the bars indicate no significant difference ($P \geq 0.01$) according to Tukey's test.

Murine Mdr3p, PfMdr1p and human MRP are all capable of transporting the **a** factor pheromone in *ste6*-deficient *S. cerevisiae*, despite significant amino acid divergence (Raymond *et al.*, 1992; Ruetz *et al.*, 1993; Volkman *et al.*, 1995). However, conclusions on substrate specificity based on heterologous expression systems should be interpreted with caution, especially with respect to MDR proteins, as it has been shown that differences in membrane composition can affect substrate specificity and ATPase activity of those proteins (Doige *et al.*, 1993; Romsicki and Sharom, 1998; Sharom, 1997). Nevertheless, the report that the half-sized LmrAp protein from *Lactococcus lactis*, the first example of a prokaryotic ABC transporter involved in MDR, can confer a typical MDR phenotype when expressed in human lung fibroblast cells, confirms that functional homology can be retained over a large evolutionary distance (Van Veen *et al.*, 1998). Thus, an understanding of evolutionary relationships among ABC transporters might help to elucidate the origins of multidrug efflux systems, their substrate specificity and their intrinsic physiological functions.

Saprophytic soil fungi such as *A. nidulans* are constantly challenged by natural toxins. By analogy to the proposed origins of bacterial multidrug transporters, we hypothesize that selection pressure has triggered the evolution of protection mechanisms based on overproduction of ABC transporters, which might originally have had a function in the transport of specific endogenous compounds (*e.g.* secondary metabolites) with the ability to expel drugs being only a fortuitous side effect (Neyfakh, 1997).

The basal level of expression of *atrC* is high as compared to that of *atrD*, which suggests an intrinsic metabolic function of AtrCp. However, the normal-growth phenotype observed for *ΔatrC* strains suggests that the role of *atrC* is not essential or can perhaps be provided by other ABC transporters. Increased transcript levels of both *atrC* and *atrD* are observed upon treatment of fungal germlings with a variety of compounds such as cycloheximide (an antibiotic), imazalil (a fungicide), pisatin (a phytoalexin from *Pisum sativum*), and reserpine (a plant indole alkaloid). This might indicate that AtrCp and AtrDp may have a function in protecting the cell against a wide range of toxic compounds. The observation that compounds can simultaneously enhance levels of both *atrC* and *atrD* transcripts suggests that both genes share similar regulatory mechanisms and even substrates. This redundancy could explain, at least in part, the finding that *ΔatrC* mutants show no hypersensitive phenotype for any of the set of compounds tested. This has also been demonstrated for single knock-out mutants of *S. cerevisiae* and *C. albicans* where

ABC transporters with distinct but overlapping drug specificities occur, making the assessment of the substrate profile of each protein only possible in multiple knock-out strains (Hirata *et al.*, 1994; Sanglard *et al.*, 1997).

The hypersensitivity observed in the *ΔatrD* mutants to the chemically unrelated compounds cycloheximide, the cyclosporin derivative PSC 833, nigericin and valinomycin provides evidence that AtrDp is involved in multidrug transport. These compounds have also been reported to be ABC-transporter substrates in other organisms (Ambudkar *et al.*, 1999; Kolaczkowski *et al.*, 1998; Kuchler *et al.*, 1989; Nishi *et al.*, 1992; Seelig, 1998). Increased sensitivity to actinomycin D as observed in *pmd1*⁻ strain of *S. pombe* was absent in Δ*atrD* strains. Several factors can account for this observation. First, an intrinsic property of the protein itself may be responsible. Second, the differences in the lipid compositions of the membranes might affect substrate specificity as discussed above. Finally, the presence of additional, as yet unknown, ABC-transporter proteins (Andrade *et al.*, 1999; Angermayr *et al.*, 1999) which share actinomycin D as substrate may compensate for the deletion of *atrD*. The latter hypothesis is supported by the observation that many ABC transporter-candidate genes are present in the expressed sequence tag (EST) database of *A. nidulans* (Roe *et al.*, 1998).

The immediate increase in accumulation of [^{14}C]fenarimol observed upon addition of reserpine suggests that this compound strongly competes with this fungicide at the substrate binding site of (a) fenarimol-efflux pump(s). The inhibitory action on [^{14}C]fenarimol efflux is transient, which might be due to rapid inactivation of reserpine (*e.g* sequestration) or to the elevated level of drug-induced efflux pump activity. The latter hypothesis is supported by the observation that reserpine strongly enhances transcription of *atrC* and, in particular, *atrD*. Fenarimol also enhances transcription of *atrD*. Therefore our results suggest that AtrDp plays a role in efflux of this fungicide. However, additional pumps involved in extrusion of fenarimol might exist as [^{14}C]fenarimol efflux activity and sensitivity to this compound in Δ*atrD* and control strains is similar. The cyclosporin derivative PSC 833, nigericin and valinomycin also induces accumulation of [^{14}C]fenarimol, but, in contrast to reserpine, their effect is not transient but proportional to time of exposure to the drug. This indicates that these compounds interfere in a different way with fenarimol efflux activity. The cyclosporin derivative PSC 833 is a strong modulator of mammalian MDR1 and therefore might have the same effect on fungal

homologs (Atadja *et al.*, 1998). The ionophores nigericin and valinomycin may also act indirectly via secondary effects (De Waard and Van Nistelrooy, 1987).

The decreased secretion of antibiotic activity of *ΔatrD* mutants suggests a role of AtrDp in penicillin secretion. This is the first report on the involvement of an ABC transporter in secretion of fungal antibiotics. In *Streptomyces peucetius* (Guilfoile and Hutchinson, 1991) and *S. argillaceus* (Fernandez *et al.*, 1996), the involvement of ABC-transporter proteins in secretion of endogenous antibiotics (*e.g.* rubicin and mithramycin, respectively) has also been demonstrated. The decrease in secreted antibiotic activity found for *ΔatrD* strains may be due to the elimination of secretion by AtrDp. However, alternative explanations are possible. AtrDp could, for instance, be involved in compartmentalization of biosynthetic precursors. In *A. nidulans*, the enzymes involved in penicillin biosynthesis are located in three different cellular compartments (Brakhage, 1998). Thus, during the biosynthesis of penicillin several transport steps are required to bring intermediates of the penicillin biosynthesis pathway together with the enzymes. If these transport steps involved AtrDp, disruption of the corresponding gene would also result in decreased penicillin production. Furthermore, AtrDp might also be part of a signal transduction mechanism that regulates some component(s) of the penicillin secretory machinery, similarly to the function proposed for the Ecs ABC-transporter proteins of *Bacillus subtilis* (Leskela *et al.*, 1999). Hence, further studies will be needed to characterize the physiological function of AtrDp in relation to penicillin biosynthesis. In addition to penicillin, *A. nidulans* is known to produce a variety of other secondary metabolites such as the hazardous carcinogen sterigmatocystin, an aflatoxin precursor. Aflatoxins are substrates of mammalian ABC transporters (Loe *et al.*, 1997). The presence of the consensus binding motif 5'-TCG(N_5)CGA-3' for AflRp, a transcription factor involved in regulation of several sterigmatocystin-biosynthesis genes (Fernandes *et al.*, 1998), in the promoter of *atrD* suggests that sterigmatocystin might be another endogenous substrate for ABC transporters in *A. nidulans*.

In summary, our results suggest that secretion of endogenous secondary metabolites, exogenous natural toxins and xenobiotics may be mediated by common ABC transporters. This may imply that strains overexpressing multidrug transporter genes can show various pleiotropic effects with respect to secretion of secondary metabolites. Such changes are of interest if they increase production of commercially important compounds. However, they may pose a danger if this would also account for detrimental compounds

such as virulence factors or mycotoxins. For these reasons, ABC transporters in *Aspergilli* need further investigation.

EXPERIMENTAL PROCEDURES

Strains, plasmids and media

The *A. nidulans* strains used in this study are listed in Table 2. All strains were derived from Glasgow stocks. Standard techniques for manipulation and growth were as described by Pontecorvo and colleagues (1953). *E. coli* DH5α was used as a host in plasmid propagation.

Table 2. *Aspergillus nidulans* strains used in this study.

Strain	Genotype[a]
Wt003	*biA1; acrA1*
WG488	*biA1; pyrG89; lysB5; fwA1; uaY9*
PAO-1 and PAO-2	Independent monosporic transformants of WG488 with plasmid pAO4-2. Prototrophic for uridine.
DC-2 and DC-7	WG488 with a single-copy replacement of *atrC* by the disruption construct of *atrC* (DC). Independent monosporic transformants.
DD-38 and DD-39	WG488 with a single-copy replacement of *atrD* by the disruption construct of *atrD* (DD). Independent monosporic transformants.

[a] For explanation of symbols, see Clutterbuck (1993).

Nucleic acids manipulations and molecular biological techniques

Freshly harvested conidia obtained from confluent plate cultures of *A. nidulans*, grown for 4-5 days at 37 ^{0}C, were used as inoculum source for liquid cultures at a density of 10^7 ml^{-1}. Germlings harvested after 14 hours of incubation at 37 ^{0}C and 200 rpm, were used for nucleic acid isolation according to Raeder and Broda (1985) and Logemann *et al.* (1987). Poly A$^+$ mRNA was purified from total RNA with the oligodex-dT^{TM} Qiagen kit (Qiagen, Chatsworth, CA, USA). cDNA synthesis was performed using the Marathon^{TM} cDNA amplification kit with the Advantage® cDNA polymerase mix (Clontech, Palo Alto, Ca, USA). The Random Primers DNA Labelling System (GIBCO BRL^{TM}, Breda, The Netherlands) was used to generate radioactively labeled oligonucleotide probes with [α-^{32}P]dATP. Southern, Northern and Dot blot hybridizations were performed using HybondN$^+$ (DNA) and HybondN (RNA) nylon membranes (Amersham), according to manufacturer's instructions. Overnight hybridized blots were washed at 65 ^{0}C with 0.1% SSC + 0.1% SDS solution. The pGEM-3Zf$^{(+)}$ and pGEM-T (Promega, Madison, Wis.) vectors were used for cloning DNA fragments and PCR products, respectively.

Sequencing was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977). PCR reactions were performed using a Perkin-Elmer DNA thermal cycler 480 and Amplitaq® DNA polymerase (Perkin Elmer, Branchburg, New Jersey, USA), unless otherwise indicated. Sequences were analyzed using the DNAstar package (DNASTAR, Inc.).

Isolation of conserved ABC motifs by PCR

The approach used was basically the same as described by Tobin *et. al.* (1997). Degenerate oligonucleotide primers were designed to amplify regions of the *A. nidulans* genome encoding consensus ABC-transporter protein sequences similar to the human MDR1, *Aureobasidium pullulans* Mdr1p and *S. cerevisiae* Ste6p. The codon bias used for primer design was based on the report of Lloyd and Sharp (1991). Primer aspmdr-1 (5'-GCYCTCGTYGGICCCTCIGG-3') or aspmdr-3 (5'-GCYCTCGTYGGICCCAGYGG-3'), encoding the amino acid sequence ALVGPSG, were used in combination with aspmdr-2 (5'-GATRCGYTGCTTYTGICCICC-3'), the complementary strand to the one encoding GGQKQRI. PCR reactions were performed at a melting temperature of 94 ^{0}C for 30 s, an annealing temperature of 60 ^{0}C for 30 s, and an extension temperature of 72 ^{0}C for 20 s and 30 cycles. Reaction products were reamplified by transferring 2 µl of the original reaction into a fresh PCR reaction mix and reamplifying under the same PCR conditions.

Genomic library

A wild-type genomic library constructed in λEMBL3 was used (Del Sorbo *et al.*, 1997). Positive lambda clones were screened and purified by at least three rounds.

Disruption constructs

The construct for disruption of *atrC* was made in three steps. First, a subclone (pC7) containing a 1.9 kb *Pst*I fragment cloned in the *Pst*I site of pGEM-3Zf$^{(+)}$ was restricted with *Bam*HI and *Bgl*II. The 3.9 kb DNA fragment was used to clone a 3.8 kb *Bam*HI insert from pAO4-2 restricted with *Bam*HI (De Ruiter Jacobs *et al.*, 1989) and named pC7O4. Second, another subclone (pC23) was restricted with *Bam*HI and a 1.2 kb *Bam*HI fragment was excised and ligated in the *Bam*HI site of pC7O4, giving rise to a 9.0 kb construct, coded pAOC. The final transformation construct, a 5.4 kb *Sph*I DNA fragment (DC), was obtained by restriction of pAOC with *Sph*I. The strategy for making the disruption

construct of *atrD* was similar. The first step consisted of cloning a 2.8 kb DNA fragment obtained from restriction of pAO4-2 with *Bam*HI and *Bgl*II into the *Bam*HI site of subclone D30, which contained a 0.4kb *Eco*RI and *Bam*HI insert. This construct was named pD30O4. In the second step, a 2.0 kb *Bgl*II fragment obtained from subclone D26 restricted with *Bgl*II was cloned in the *Bam*HI site of pD30O4, resulting in a 8.4 kb construct coded pAOD. The final transformation construct of 5.2 kb was obtained by the restriction of pAOD with *Xho*I and *Eco*RI.

Preparation of protoplasts and transformation

Mycelial protoplasts were prepared as described by Wernars *et. al.* (1985) with minor modifications. Liquid minimal medium supplemented with 2 g l^{-1} casaminoacids, 0.5 g l^{-1} yeast extract and auxotrophic markers was inoculated with 10^6 conidia ml^{-1} and incubated overnight at 37 ^{0}C and 300 rpm in a orbital incubator for 16 hours. The germlings were harvested through Mira-Cloth, washed twice with sterile water and twice with STC buffer (1.0 M sorbitol, 10mM Tris-Cl pH 7.5, 50 mM $CaCl_2$) and squeezed between paper towels to remove excess of liquid. Protoplasts were released by incubation of one gram of mycelium at 30 ^{0}C and 100 rpm, resuspended in 20 ml of filter-sterilized iso-osmotic S0.8MC medium containing lytic enzymes (5 mg ml^{-1} Novozym 234, 0.8 M KCl, 50 mM $CaCl_2$, 20 mM MES pH 5.8) for about 2 hours. The protoplast solution was filtered over glass-wool, diluted (1:1) with STC buffer and incubated on ice for 10 min. Then, protoplasts were collected by centrifugation (10 min, 0 ^{0}C, 3000 rpm) and washed twice with STC buffer. Transformation was performed as described by Van Heemst *et al.* (1997) using purified DNA (3.5 µg) of transformation constructs DC and DD dissolved in sterile water (15 µl).

Toxicity assays

Sensitivity of *A. nidulans* strains to toxicants was determined in a radial growth test on MM plates (De Waard and Van Nistelrooy, 1979). Benomyl and sulfomethuron methyl were kindly provided by DuPont De Nemours (Wilmington, USA), bitertanol by Bayer AG (Leverkusen, Germany), cilofungin by Eli Lilly and Co. (Indianapolis, USA), the cyclosporin derivative PSC 833 by Novartis (Basel, Switzerland), fenarimol by Dow Elanco (Greenfield, USA) and imazalil nitrate by Janssen Pharmaceuticals (Beerse, Belgium). Pisatin was purified from pea pods (Fuchs *et al.*, 1981). All other chemicals

tested were purchased from Sigma Chemical Co. (Zwijndrecht, The Netherlands). Final concentrations of test chemicals in agar were: actinomycin D (100 μg ml^{-1}), benomyl (1 μg ml^{-1}), bergenin (100 μg ml^{-1}), bitertanol (10 μg ml^{-1}), cycloheximide (25 μg ml^{-1}), cyclosporin derivative PSC 833 (25 μg ml^{-1}), chlorpromazine (25 μg ml^{-1}), chloramphenicol (100 μg ml^{-1}), eugenol (2.5 μl ml^{-1}), fenarimol (3 μg ml^{-1}), genistein (100 μg ml^{-1}), gramicidin D (10 μg ml^{-1}), imazalil (0.03 μg ml^{-1}), kresoxim methyl (0.3 μg ml^{-1}), nigericin (3 μg ml^{-1}), nystatin (10 μg ml^{-1}), 4-nitroquinoline-*N*-oxide (1 μg ml^{-1}), oligomycin (0.25 μg ml^{-1}), pisatin (20 μg ml^{-1}), quinidine (200 μg ml^{-1}), resveratrol (200 μg ml^{-1}), rhodamine 6G (5 μg ml^{-1}), triflupromazine (10 μg ml^{-1}), tomatine (10 μg ml^{-1}), valinomycin (3 μg ml^{-1}). These compounds were added from concentrated solutions in methanol. Amphotericin B (25 μg ml^{-1}), brefeldin A (5 μg ml^{-1}), camptothecin (25 μg ml^{-1}), cilofungin (0.1 μg ml^{-1}), psoralen (200 μg ml^{-1}), quercetin (200 μg ml^{-1}), sulfomethuron methyl (100 μg ml^{-1}) were added from concentrated solutions in DMSO. Acriflavin (1 μg ml^{-1}), ethidium bromide (1 μg ml^{-1}) and neomycin sulphate (600 μg ml^{-1}) were dissolved in sterile water. The final concentration of the solvents in all treatments never exceeded 1%.

Accumulation of [^{14}C]fenarimol

Experiments were performed with standard suspensions of germlings of *A. nidulans* at an initial external concentration of 30 μM [^{14}C]fenarimol (De Waard and Van Nistelrooy, 1980).

Bioassays

A. nidulans strains were point-inoculated on agar plates containing complete medium (CM) and incubated for 14 days at 25 ^{0}C. The strain of *M. luteus* (DSM-348) was purchased from DSMZ (Braunschweig, Germany). Overnight bacterial cultures were grown on Lab-Lemco Broth (Oxoid) at 30 ^{0}C and 200 rpm. In bioassays, portions (50 ml) of freshly prepared sterile Lab-Lemco Agar (Oxoid) were cooled-down (45 ^{0}C), mixed with overnight bacterial culture (1 ml) and transferred to 145 mm (diameter) plates. Then, agar plugs from the center of 14-day-old colonies of *A. nidulans* strains to be tested were placed equidistantly on top of the bacterial plates and incubated at 30 ^{0}C. Inhibition zones were measured after 24 hours of incubation.

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Chapter 4

The ABC transporter AtrB from *Aspergillus nidulans* mediates resistance to all major classes of fungicides and some natural toxic compounds

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SUMMARY

This paper reports the functional characterization of AtrBp, an ABC transporter from *Aspergillus nidulans*. AtrBp is a multidrug transporter and has affinity to substrates belonging to all major classes of agricultural fungicides and some natural toxic compounds. The substrate profile of AtrBp was determined by assessing the sensitivity of deletion and overexpression mutants of *atrB* to several toxicants. All mutants showed normal growth as compared to control isolates. Δ*atrB* mutants displayed increased sensitivity to anilinopyrimidine, benzimidazole, phenylpyrrole, phenylpyridylamine, strobilurin, and some azole fungicides. Increased sensitivity to the natural toxic compounds camptothecin (alkaloid), the phytoalexin resveratrol (stilbene) and the mutagen 4-nitroquinoline-*N*-oxide was also found. Overexpression mutants were less sensitive to a wide range of chemicals. In addition to the compounds mentioned above, decreased sensitivity to a broader range of azoles, dicarboximides, quintozene, acriflavine and rhodamine 6G was observed. Decreased sensitivity in overexpression mutants negatively correlated with levels of *atrB* expression. Interestingly, the overexpression mutants displayed increased sensitivity to dithiocarbamates fungicides, chlorothalonil and the iron activated antibiotic phleomycin. Accumulation of the azole fungicide [^{14}C]fenarimol by the overexpression mutants was lower as compared to the parental isolate, demonstrating that AtrBp acts by preventing intracellular accumulation of the toxicant. Various metabolic inhibitors increased accumulation levels of [^{14}C]fenarimol in the overexpression mutants to wild-type levels, indicating that reduced accumulation of the fungicide in these mutants is due to increased energy-dependent efflux as a result of higher pump capacity of AtrBp.

INTRODUCTION

The use of fungicides in crop production continues to be essential for effective control of plant diseases and assurance of high crop yields. Recently, new classes of fungicides have been developed in order to meet the public demand of environmentally safer products. These modern fungicides include the classes of anilinopyrimidines, phenoxyquinolines, phenylpyrroles and strobilurins (Knight *et al.*, 1997), which are highly selective site-specific inhibitors of the metabolism of target organisms. A disadvantage of fungicides with a specific mode of action is the high risk of resistance development (Jespers and De

Waard, 1993). This has been the case for the first generation of modern fungicides (*e.g.* benzimidazoles, phenylamides, dicarboximides, and sterol biosynthesis inhibitors), whose activity was significantly reduced by the development of resistance in target populations. In some cases, resistance developed not only to a specific fungicide but also to structurally and functionally unrelated compounds. This phenomenon, known as **multidrug** resistance (MDR), has been reported to operate in a broad range of organisms and is of major concern in clinical medicine. Therefore, understanding the mechanisms of multidrug resistance development is important for society in general.

A common mechanism of MDR is the overexpression of energy-dependent multidrug efflux pumps, also known as multidrug transporter proteins or P-glycoproteins (P-gp). Overexpression of such proteins in cancer cells results in MDR to chemotherapeutic drugs and other hydrophobic pharmacological agents (Ambudkar *et al.*, 1999). P-glycoproteins belong to the ubiquitous superfamily of **A**TP-**B**inding **C**assette (ABC) transporters. Besides multidrug transporters, the family includes proteins involved in transmembrane transport of various substances such as ions, amino acids, peptides, sugars, vitamins, steroid hormones, bile acids and phospholipids (Higgins, 1992, 1994; Van Helvoort *et al.*, 1996).

In filamentous fungi, MDR was first reported for laboratory-generated mutants of *Aspergillus nidulans* selected for resistance to azole fungicides, also described as sterol biosynthesis inhibitors (Van Tuyl, 1977). Resistance to azoles in isogenic mutants is based on an energy-dependent efflux mechanism which results in decreased accumulation of compounds in fungal mycelium, similarly to the phenomenon observed in cancer cells (De Waard and Van Nistelrooy, 1979, 1980). This mechanism also operates in plant pathogens such as *Penicilium italicum*, *Botrytis cinerea*, *Nectria haematococca*, and probably *Mycosphaeaerella graminicola* (De Waard *et al.*, 1996; Joseph-Horne *et al.*, 1996). To date, at least five ABC transporters highly homologous to multidrug-efflux pumps from other organisms have been described for *A. nidulans* (Andrade *et al.*, 1999; Angermayr *et al.*, 1999; Del Sorbo *et al.*, 1997).

This paper describes the functional characterization of *atrB*, a previously described gene of *A. nidulans* (Del Sorbo *et al.*, 1997). AtrBp displays a high degree of sequence homology to BcatrBp from *B. cinerea*, Mgatr5p from *M. graminicola*, Pmr1p from *Penicillium digitatum*, and Abc1p from *Magnaporthe grisea* (Goodall *et al.*, 1999; Nakaune *et al.*, 1998; Schoonbeek *et al.*, 1999; Urban *et al.*, 1999). A high degree of homology also exists with ABC proteins classified in sub-cluster I.1 from *Saccharomyces*

cerevisiae (Decottignies and Goffeau, 1997), Bfr1p from *Schizosaccharomyces pombe* (Nagao *et al.*, 1995), and the Cdr1p and Cdr2p proteins from *Candida albicans* (Prasad *et al.*, 1995; Sanglard *et al.*, 1996, 1997). Most of these proteins have been characterized as multidrug-efflux pumps. Previously, we have reported that heterologous overexpression of *atrB* in *S. cerevisiae* restores wild-type sensitivity to cycloheximide, tentatively indicating that AtrBp is also a multidrug-efflux protein. Here, we describe in detail the substrate specificity of the multidrug transporter AtrBp by phenotype characterization of knock-out and overexpression mutants of *A. nidulans* with respect to fungicide sensitivity. Δ*atrB* strains display increased sensitivity to several classes of fungicides and some natural toxic compounds. *atrB* overexpression mutants are less sensitive to a wide range of compounds. Interestingly, these overexpression mutants display at the same time increased sensitivity to some conventional fungicides and phleomycin, an iron-activated antibiotic. These results clearly indicate that AtrBp is a multidrug transporter involved in protection against natural toxins and xenobiotics and might play a role in iron metabolism.

RESULTS

Generation of Δ*atrB* mutants

To characterize the substrate specificity of AtrBp and its role in MDR, we have generated deletion mutants by replacing the major part of the coding region of *atrB* with the orotidine-5'-phosphate decarboxylase (*pyrG*) encoding-gene of *A. oryzae*. The uridine-auxotrophic strain WG488 of *A. nidulans* was used as the recipient strain for transformation and selection of transformants was based on uridine prototrophy. The use of the heterologous selectable marker of *A. oryzae* minimizes the chance of integration of the construct at the *pyrG* locus of *A. nidulans*. The schematic representation of the disruption strategy used is given in Fig. 1(a). Southern blot analysis confirmed replacement of the wild-type allele of *atrB* in all mutants tested (Fig. 1b). The replacement of the *atrB* locus was confirmed by the expected shift in size of the restriction fragments when the blots were hybridized with probe B1 (left panel). When the same blots were hybridized with probe B2, a smaller hybridizing fragment as compared to the wild-type locus (Lane 1, right panel) was expected to occur (Lanes 2-9). However, in some of the mutants (Lanes 2, 3 and 6) a restriction fragment of the same size as the wild-type locus (Lane 1) was still present and indicated that these mutants are heterokaryons. For further analysis two independent mutants DB5 (Fig. 1b; lane 4) and DB21 (Fig. 1b; lane 9) were selected.

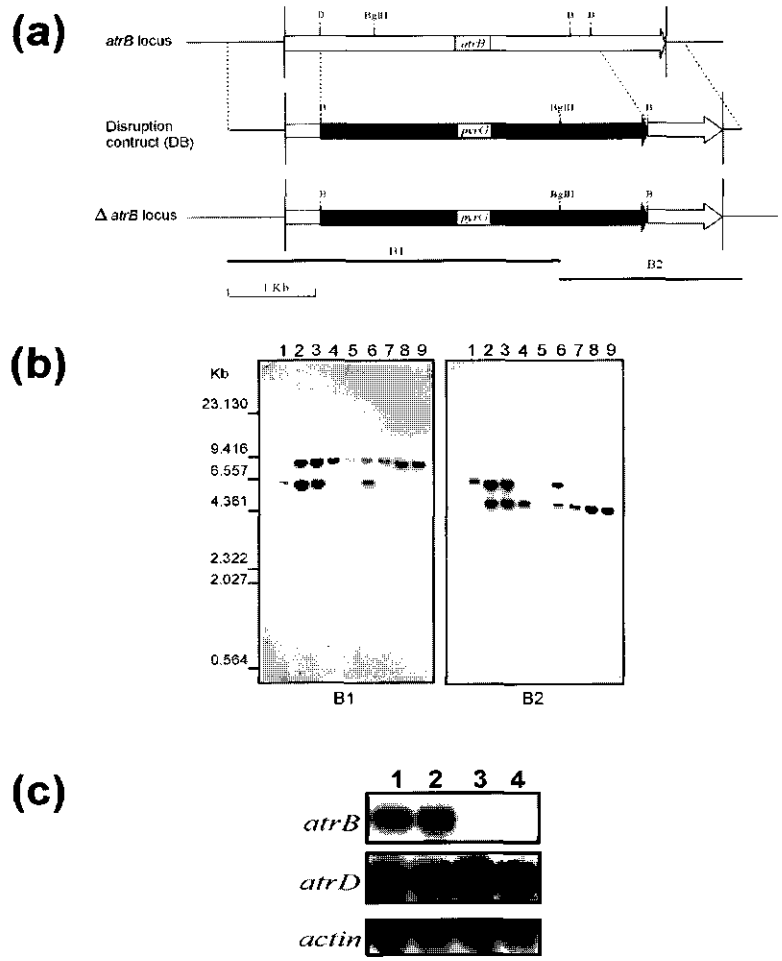

Figure 1. Generation of deletion mutants of the *Aspergillus nidulans atrB* gene
(a) Schematic representation of the wild-type *atrB* locus, disruption construct, and knock-out locus of *atrB*. Horizontal lines labeled B1 and B2 indicate the restriction fragments used as probes in Southern analysis. **(b)** Southern blot analysis was performed with genomic DNA from the recipient strain WG488 used for transformation (Lane 1) and eight putative Δ*atrB* isolates (Lanes 2-9). Genomic DNA of WG488, and DB isolates was restricted with *BglII* and hybridized with probes B1 and B2 (left and right panels, respectively). **(c)** Northern blot analysis of RNA isolated from germlings treated with cycloheximide (20 μg ml^{-1}) for 60 min. The lanes represent the control strains PAO-1 (Lane 1) and PAO-2 (Lane 2) transformed with the pAO-2 vector containing the *pyrG* gene of *A. oryzae* and the Δ*atrB* strains DB5 (Lane 3) and DB21 (Lane 4). The upper panel shows the result of a hybridization with the gene-specific probe D15 of *atrB* (Del Sorbo *et al.*, 1997). The middle panel show the same blot hybridized with the gene-specific probe GspD of *atrD* and the bottom panel with actin as loading controls.

Northern blot analysis was carried out with total RNA isolated from germlings treated with cycloheximide, a strong inducer of *atrB* transcript levels. This treatment did not reveal any signal of mRNA from *atrB* in the Δ*atrB* mutants, whereas transcript levels of *atrD* were the same in all strains tested (Fig. 1c). These observations confirm that *atrB* was functionally deleted.

Generation of overexpression mutants of *atrB*

Generation of mutants with increased copy number of *atrB* was achieved by transformation of strain WG488 with construct pOB, which contains a genomic copy of *atrB* comprising the coding region plus the 5' and 3'untranslated regions (UTR). This construct was cloned in the pPL6 vector (Oakley *et al.*, 1987), which contains the *pyrG* from *A. nidulans*. A schematic representation of the transformation construct coded pOB is presented in Fig. 2(a). After selection of transformants based on uridine prototrophy, sensitivity to camptothecin was tested. This compound was selected because Δ*atrB* mutants displayed a strong increase in sensitivity to this compound as compared to control isolates (Fig. 2c, left panel). Therefore, increase in the copy number of *atrB* should lead to decreased sensitivity to camptothecin. Using this screening procedure, we isolated mutants with different levels of resistance to camptothecin, as compared to the control isolates (data not shown). We postulated that this differential degree of resistance could be due to different copy number of *atrB*. To investigate this assumption, mutants displaying different levels (low, intermediate and high) of resistance to camptothecin were selected for further characterization (OB7, OB16 and OB35, respectively). Southern blot analysis confirmed an increase in *atrB* copy number in all strains tested (data not shown) and Northern blot analysis confirmed that sensitivity to camptothecin in the different overexpression mutants was negatively correlated with levels of *atrB* transcription (Fig. 2b and right panel of 2c).

Phenotype characterization

Two independent monospore strains of Δ*atrB*, DB5 and DB21 were selected for phenotype characterization with respect to sensitivity to fungicides and other toxicants. Strains PAO-1 and PAO-2, transformed with a construct containing the *pyrG* from *A. oryzae* only, were used as controls. The deletion mutants grew normally and no differences in radial growth rates were observed. A radial growth toxicity test was used to evaluate the role of AtrBp in

drug sensitivity. The activity of 50 compounds (see Experimental procedures) was tested. Δ*atrB* mutants displayed increased sensitivity to the fungicides azoxystrobin, camptothecin, carbendazim, cyprodinil, fenpiclonil, fludioxonil, fluazinam, ketoconazole, kresoxim-methyl, 4-nitroquinoline oxide, prochloraz, propiconazole, resveratrol, thiabendazole, trifloxystrobin, when compared to the control isolates tested (Fig. 3a, Tables 1 and 2). Δ*atrB* mutants did not display increased sensitivity to cycloheximide, although we have shown previously that *atrB* was able to confer decreased sensitivity to this compound when overexpressed in yeast cells (Del Sorbo *et al.*, 1997).

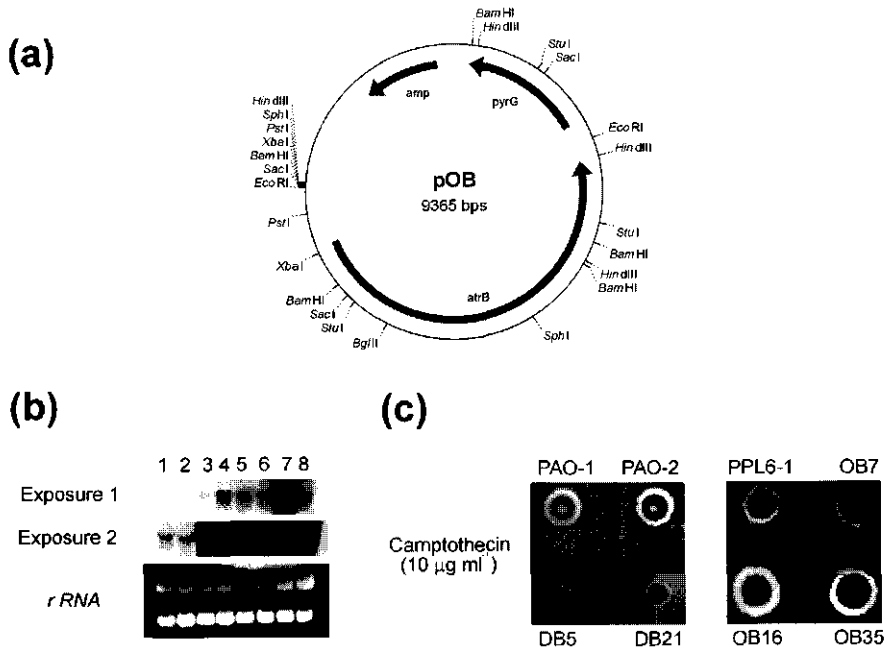

Figure 2. Generation of overexpression mutants of the *Aspergillus nidulans atrB* gene
(a) Schematic representation of the pOB construct used for transformation. Restriction sites are indicated in italics. **(b)** Northern blot analysis of RNA isolated from untreated germlings of *A. nidulans* after different exposure times of blots. The lanes contain RNA samples obtained from the control strains PPL6-1 (Lane 1) and PPL6-2 (Lane 2) and the *atrB* overexpression mutants OB7 (Lane 3), OB16 (Lane 4), OB18 (Lane 5), OB30 (Lane 6), OB35 (Lane 7) and OB44 (Lane 8). Bottom panel: loading control (ethidium bromide stained blot). **(c)** Altered sensitivity to camptothecin displayed by *atrB* deletion (DB-5 and DB-21) and overexpression (OB-7, OB-16 and OB-35) mutants as compared to control strains (PAO and PPL6-1, respectively).

Sensitivity of the overexpression mutants OB7, OB16 and OB35 to the same set of compounds described above was also determined. Strain PPL6-1 transformed with the pPL6 construct was used as control strain. The overexpression mutants displayed decreased sensitivity to a wide range of compounds (Tables 1 and 2). Furthermore, the degree of decreased sensitivity to these compounds was positively correlated with the level of *atrB* expression (Fig. 3b, Tables 1 and 2). Most interestingly, we also observed that the overexpression mutants displayed increased sensitivity to compounds such as chlorothalonil, ferbam, thiram, and phleomycin. The increased sensitivity was negatively correlated with the level of *atrB* expression (Fig. 3c, Tables 1 and 2).

AtrBp causes energy-dependent efflux of [^{14}C]fenarimol

In genetically defined MDR mutants of *A. nidulans*, resistance to the azole fungicide fenarimol is based on increased energy-dependent efflux activity which results in decreased cytoplasmic drug accumulation (De Waard and Van Nistelrooy, 1979, 1980). We could not find any significant difference in [^{14}C]fenarimol accumulation between the control PAO and the Δ*atrB* strains (Fig. 4a). However, initial [^{14}C]fenarimol accumulation in *atrB* overexpression mutants was lower than in control PPL6-1 strain (Fig. 4b). In radial growth tests, mutants overexpressing *atrB* had decreased sensitivity to fenarimol.

We also confirmed that the efflux mechanism operating in the overexpression mutant OB35, as in the control strain PPL6-1, was energy-dependent. This conclusion is based on results of experiments in which the effect of respiratory inhibitors (oligomycin and CCCP) and an inhibitor of membrane ATPases (*ortho*-vanadate) on accumulation of [^{14}C]fenarimol was tested. Addition of these compounds instantly increased accumulation of [^{14}C]fenarimol (Fig. 4c). This effect is ascribed to inhibition of energy-dependent [^{14}C]fenarimol efflux activity as reported previously (De Waard and Van Nistelrooy, 1980). In the same way, we have checked the potency of two substrates (kresoxim-methyl and iprodione) of AtrBp, to competitively inhibit the efflux of [^{14}C]fenarimol. These compounds also stimulate the accumulation of [^{14}C]fenarimol. The inhibitory activity of iprodione seems to be transient in time (Fig. 4d).

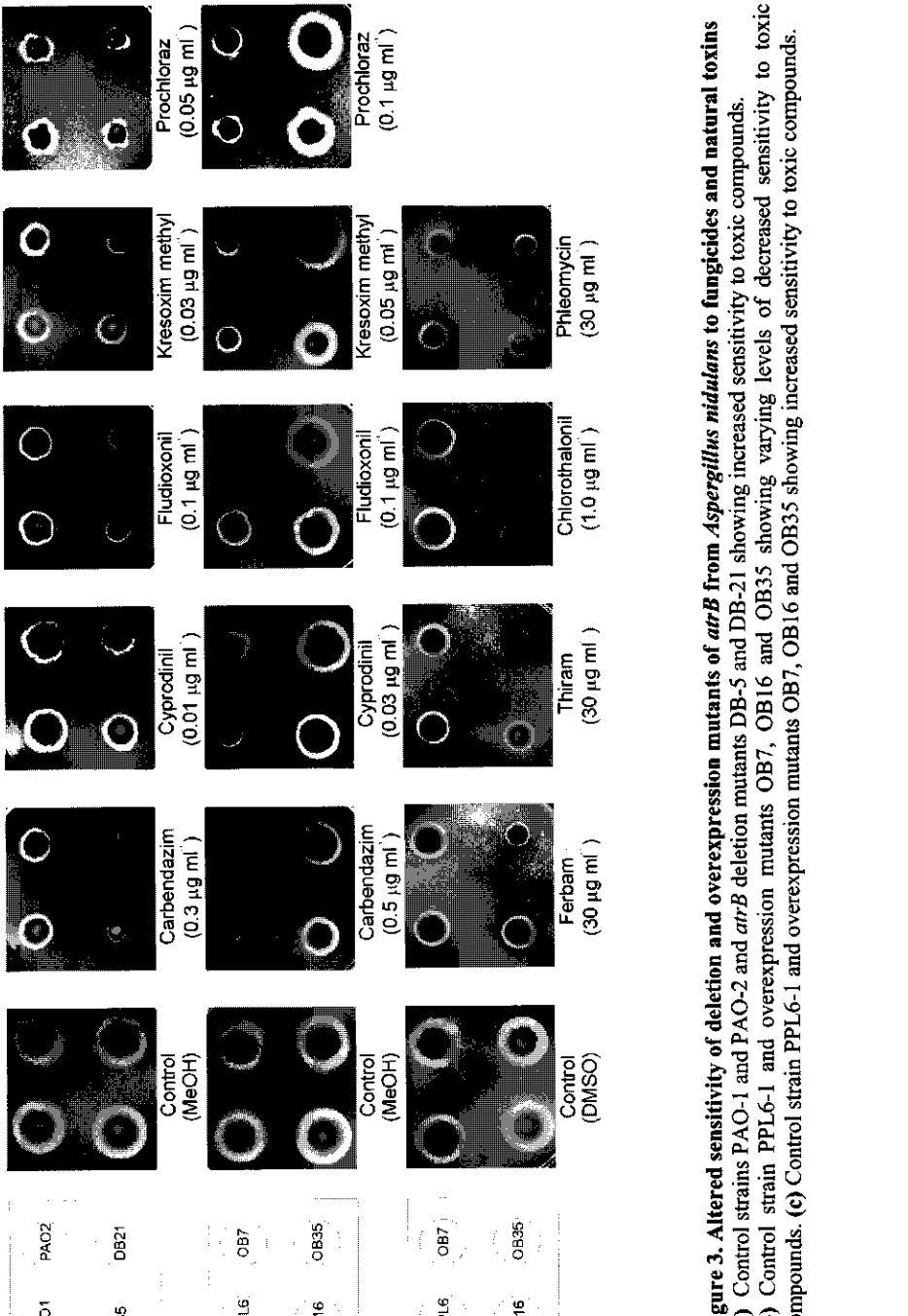

Figure 3. Altered sensitivity of deletion and overexpression mutants of *atrB* from *Aspergillus nidulans* to fungicides and natural toxins
(a) Control strains PAO-1 and PAO-2 and *atrB* deletion mutants DB-5 and DB-21 showing increased sensitivity to toxic compounds.
(b) Control strain PPL6-1 and overexpression mutants OB7, OB16 and OB35 showing varying levels of decreased sensitivity to toxic compounds. **(c)** Control strain PPL6-1 and overexpression mutants OB7, OB16 and OB35 showing increased sensitivity to toxic compounds.

Table 1. Sensitivity of deletion (DB5 and DB21) and overexpression mutants (OB7, OB16 and OB35) of *atrB* in *Aspergillus nidulans* to fungicides and antimycotics in radial growth tests.

Fungicides and antimycotics	EC_{50} Pao* ($mg\ l^{-1}$)	Q_{50}†	EC_{50} Ppl6* ($mg\ l^{-1}$)	Q_{50}†		
		ΔatrB		OB7	OB16	OB35
Azoles						
Fenarimol	2.32	0.97	2.89	1.46	1.81	2.34 ‡
Imazalil	0.04	0.92	0.05	1.00	1.19	1.66 ‡
Itraconazole	0.05	0.94	0.05	0.91	0.98	1.00
Ketoconazole	0.12	0.71 ‡	0.07	1.08	1.27	1.93 ‡
Miconazole	0.36	1.03	0.33	1.42	1.74	3.51 ‡
Prochloraz	0.08	0.72 ‡	0.06	1.41 ‡	>10 ‡	>100 ‡
Propiconazole	1.23	0.63 ‡	1.45	1.00	1.34 ‡	2.28 ‡
Anilinopyrimidines						
Cyprodinil	0.03	0.45 ‡	0.02	1.60 ‡	3.14 ‡	4.16 ‡
Pyrimethanil	0.18	0.92	0.20	1.00	1.26	1.50 ‡
Aromatic hydrocarbons						
Na-*o*-phenylphenate	12.41	1.02	12.67	0.95	0.95	0.91
Quintozene	7.42	0.94	6.73	1.00	1.50	6.33 ‡
Benzimidazoles						
Carbendazim	0.22	0.86 ‡	0.23	1.23 ‡	1.77 ‡	1.88 ‡
Thiabendazole	1.99	0.93	1.78	1.15 ‡	1.51 ‡	2.21 ‡
Dicarboximides						
Iprodione	2.93	0.95	2.64	1.00	1.24	1.79 ‡
Vinclozolin	4.92	0.96	5.78	1.00	1.28 ‡	1.67 ‡
Dithiocarbamates						
Ferbam	31.21	0.93	29.17	1.00	0.96	0.74 ‡
Thiram	25.41	1.00	28.78	0.93	0.88	0.55 ‡
Phenylpyrroles						
Fenpiclonil	0.15	0.87 ‡	0.15	1.50	1.85 ‡	5.62 ‡
Fludioxonil	0.10	0.49 ‡	0.10	1.67 ‡	3.74 ‡	6.00 ‡
Phenylpyridylamines						
Fluazinam	0.20	0.33 ‡	0.13	1.00	1.37 ‡	1.72 ‡
Strobilurins						
Azoxystrobin	0.04	0.49 ‡	0.02	1.60 ‡	2.07 ‡	7.13 ‡
Kresoxim-methyl	0.05	0.73 ‡	0.04	1.42 ‡	2.48 ‡	7.42 ‡
Trifloxystrobin	0.002	0.59 ‡	0.002	1.00	3.55 ‡	5.57 ‡
Polyene antibiotics						
Amphotericin B	58.0	0.86	52.8	0.86	0.87	0.79
Nystatin	13.1	0.76	17.6	1.00	0.84	0.58 ‡
Miscellaneous						
Cilofungin	0.02	1.02	0.02	0.92	1.07	1.09
Chlorothalonil	1.68	1.35	1.46	1.07	0.86	0.12 ‡

* Control strains

† Degree of sensitivity expressed as EC_{50} mutant/EC_{50} control strain.

‡ Mean values of colony size of control strains and mutants growing on agar amended with fungicides around EC_{50} concentrations (see exp. procedures) are statistically different according to Tukey's test ($P<0.05$).

DISCUSSION

We have shown that deletion and overexpression mutants of *atrB* in *A. nidulans* display differential sensitivity to structurally unrelated compounds. These results indicate that the ABC transporter AtrBp is a multidrug transporter and accepts these compounds as substrates. We propose that the reduced intracellular accumulation of [^{14}C]fenarimol in overexpression mutants of *atrB* and the decreased sensitivity to fenarimol can be explained by increased efflux activity of the fungicide.

Table 2. Sensitivity of deletion (DB5 and DB21) and overexpression mutants (OB7, OB16 and OB35) of *atrB* in *Aspergillus nidulans* to natural toxic products and miscellaneous compounds in radial growth tests.

Compounds	EC_{50} Pao* (mg l^{-1})	Q_{50}†	EC_{50} Ppl6* (mg l^{-1})	Q_{50}†		
		Δ*atrB*		OB7	OB16	OB35
Antibiotics						
Cycloheximide	63.8	1.07	71.9	0.91	1.00	0.91
Phleomycin	31.1	1.04	28.3	0.71	0.56 ‡	0.35 ‡
Ionophores						
Nigericin	3.3	1.01	3.2	1.01	1.02	1.01
Miscellaneous						
Acriflavine	1.95	0.80	1.43	1.16	2.78 ‡	6.07 ‡
4-NQO	1.65	0.69	1.50	0.97	1.60 ‡	1.67 ‡
Rhodamine 6G	4.74	0.90	4.35	1.06	1.20	2.24 ‡
CCCP	0.40	0.76	0.32	1.12	1.23	1.24 ‡
Plant compounds						
Camptothecin	65.3	0.24 ‡	66.7	- §	- §	- §
Eugenol	140	0.80	170	0.92	0.95	1.00
Resveratrol	- §	< 1 ‡	- §	- §	- §	- §

* Control strains
† Degree of sensitivity expressed as EC_{50} mutant/EC_{50} control strain.
‡ Mean values of colony size of control strains and mutants growing on agar amended with fungicides around EC_{50} concentrations (see methods) are statistically different according to Tukey's test ($P<0.05$).
§ EC_{50} values for the mutants are above solubility level of the compound and could not be accurately determined.

Deletion strains of *atrB* displayed increased sensitivity to different classes of agricultural fungicides: cyprodinil (anilinopyrimidine), ketoconazole, prochloraz and propiconazole (azoles), carbendazim (benzimidazole), fenpiclonil and fludioxonil (phenylpyrroles), fluazinam (phenylpyridilamine) and azoxystrobin, kresoxim-methyl and trifloxystrobin (strobilurins). Increased sensitivity was also observed for other

compounds such as 4-nitroquinoline-*N*-oxide (mutagen), camptothecin (plant alkaloid), and the phytoalexin resveratrol (stilbene). These results indicate that these compounds are substrates of AtrBp.

Analogous to ABC transporters of yeast (Kolaczkowski *et al.*, 1998; Sanglard *et al.*, 1996; Sanglard *et al.*, 1997), ABC transporters of *A. nidulans* may have distinct but overlapping substrate specificities. This makes it difficult to assess the substrate profile of an ABC protein using single knock-out mutants. To overcome this problem, the sensitivity of overexpression mutants to toxicants was also determined. This approach led to the characterization of additional substrates, such as fenarimol, imazalil and miconazole (azoles), pyrimethanil (anilinopyrimidine), iprodione and vinchlozolin (dicarboximides), quintozene (aromatic fungicides), acriflavine and rhodamine 6G. In all cases, an inverse correlation between levels of *atrB* expression in the overexpression mutants and sensitivity to toxicants was established. These results provide evidence that AtrBp pump activity is responsible for the decreased sensitivity to toxicants. The results also imply that the use of overexpression mutants avoids or minimizes the problem of redundancy of ABC transporters in characterization of the substrate specificity of AtrBp. Phenotype characterization of multiple deletion mutants is another approach that can be used to minimize the problem of redundancy. This approach was used to characterize the drug-resistance profile of the major ABC transporters of the PDR network from *S. cerevisae* (Kolaczkowski *et al.*, 1998). The sensitivity of isogenic *S. cerevisae* strains deleted in *PDR5*, *SNQ2*, or *YOR1*, and multiple knock-outs in different combinations was tested to 349 toxic compounds. Several fungicides, similar to the ones used in our study, appeared to be ABC-transporter substrates in that organism.

The transient accumulation of [^{14}C]fenarimol in the *ΔatrB* mutants and control strains is similar. In contrast, the overexpression mutants have a lower initial level of [^{14}C]fenarimol accumulation. These results indicate that AtrBp can act as a fenarimol efflux pump. However, results also suggest that *A. nidulans* has (an) additional efflux pump(s) accepting fenarimol as substrate. In *ΔatrB* mutants, such additional efflux pumps may compensate for the absence of AtrBp, resulting in similar patterns of [^{14}C]fenarimol accumulation. Such compensating efflux pumps are still unknown but it might be one of the many ABC-transporter-candidate genes present in the expressed sequence tag (EST) database of *A. nidulans* (Roe *et al.*, 1998).

Restoration of wild-type levels of [^{14}C]fenarimol accumulation in the overexpression mutant OB35 after addition of the respiratory inhibitors (CCCP and oligomycin) and an inhibitor of membrane ATPases (*ortho*-vanadate), demonstrates that the [^{14}C]fenarimol efflux is energy-dependent. This may be due to a direct effect of the inhibitor on the AtrB protein (vanadate), an effect on ATP synthesis in mitochondria (CCCP, oligomycin), and indirectly via dissipation of the proton-motive force (CCCP). Furthermore, identified substrates in the toxicity assays such as kresoxim-methyl and iprodione, also stimulate accumulation of [^{14}C]fenarimol, suggesting that these compounds are competitive inhibitors of [^{14}C]fenarimol efflux. Interestingly, a different pattern of inhibition for the two compounds was observed. First, the iprodione concentration (300 μM) required to increase [^{14}C]fenarimol accumulation was ten times higher than the one used for fenarimol (30 μM). Kresoxim-methyl showed this effect at equimolar concentrations (30 μM). This suggests that AtrBp has a higher affinity for kresoxim-methyl than for iprodione. Altered sensitivity to iprodione was only detected in the overexpression mutants whilst altered sensitivity to kresoxim-methyl was detected in both deletion and overexpression mutants of *atrB*. These results also suggest that AtrBp has a relative high-affinity to kresoxim-methyl.

Similarly to the yeast ABC-transporter proteins of sub-cluster I.1 (Decottignies and Goffeau, 1997), AtrBp has the $(NBF\text{-}TMD)_2$ configuration. The majority of ABC transporters involved in MDR from yeast, such as Pdr5p, Snq2p and Pdr12p are grouped in this sub-cluster. Genes encoding proteins with very high homology to AtrBp have been described for at least two important plant pathogens, *B. cinerea* and *M. graminicola* (Goodall *et al.*, 1999; Schoonbeek *et al.*, 1999). A BLAST analysis with the AtrBp sequence reveals that BcatrBp from *B. cinerea* is its closest homologue with an overall identity of 70%. Most interestingly, the predicted transmembrane domains of both proteins are also highly conserved. This suggest that BcatrBp from *B. cinerea* may have similar substrates as AtrBp from *A. nidulans*.

Wild-type sensitivity to cycloheximide was restored to the *PDR5*-deficient strain, upon transformation with the cDNA of *atrB* in a high-copy-number vector (Del Sorbo *et al.*, 1997). In the present work, neither *ΔatrB* nor overexpression mutants of *A. nidulans* displayed altered sensitivity to cycloheximide as compared to the control strains. It has been demonstrated for the human MDR1 protein that lipid composition of membranes can affect its substrate specificity and ATPase activity (Doige *et al.*, 1993;

Romsicki and Sharom, 1998; Sharom, 1997). Hence, differences in membrane composition of yeast as compared to *A. nidulans* could explain these results.

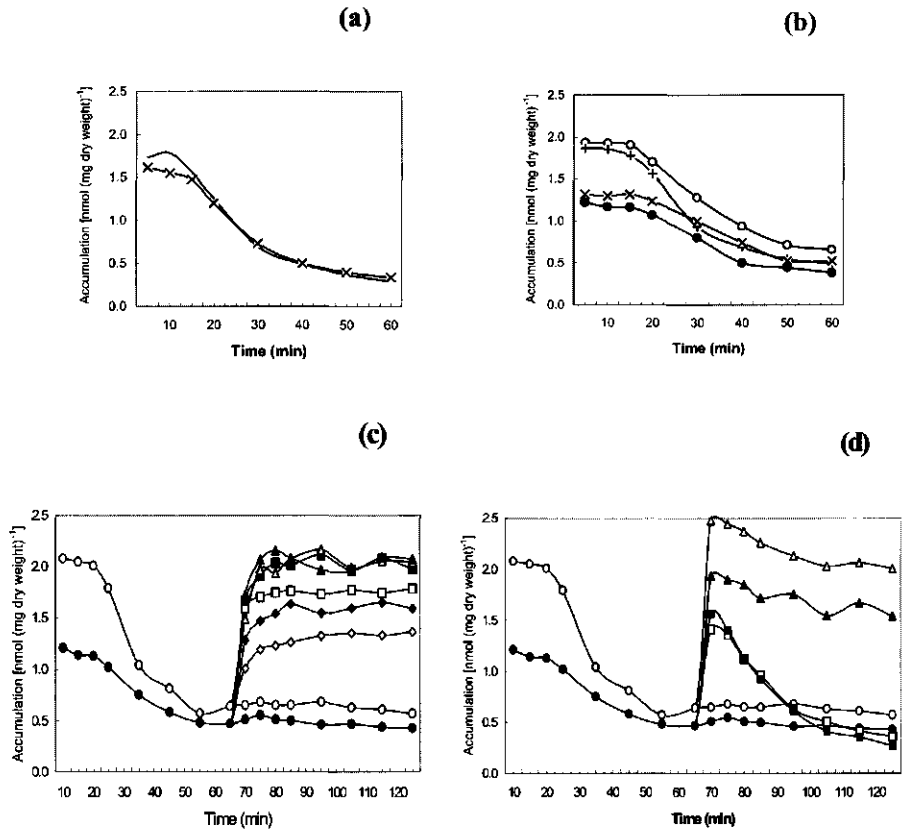

Fig. 4. Accumulation of [^{14}C]fenarimol by germlings of *Aspergillus nidulans*
Accumulation of [^{14}C]fenarimol (30 µM) by control strains PAO (bold line) and Δ*atrB* mutants (x). **(b)** Accumulation of [^{14}C]fenarimol by control strain PPL6-1 (○) and the *atrB* overexpression mutants OB7 (+), OB16 (X) and OB35 (●). **(c)** Effect of CCCP (□, ■), oligomycin (△, ▲) and sodium ortho-vanadate (◇, ◆) on [^{14}C]fenarimol accumulation by control strain PPL6-1 (open symbols) and the *atrB* overexpression mutant OB35 (filled symbols). CCCP (30 µM), oligomycin (30 µM) and sodium ortho-vanadate (30 mM) added 60 min after addition of [^{14}C]fenarimol (t=0). Controls: methanol (0.1 %; ○, ●). **(d)** Effect of kresoxim-methyl (△, ▲) and iprodione (□, ■) on [^{14}C]fenarimol accumulation by germlings of the control strain PPL6-1 (open symbols) and the *atrB* overexpression mutant OB35 (filled symbols). Kresoxim-methyl (30 µM) and iprodione (300 µM) added 60 min after addition of [^{14}C]fenarimol (t=0). Controls: methanol (0.1 %; ○, ●).

Most interestingly, the overexpression mutants of *atrB* displayed increased sensitivity to dithiocarbamates fungicides, chlorothalonil and the iron-activated antibiotic phleomycin. The increase in sensitivity of the overexpression mutants negatively correlated with the levels of *atrB* expression in the different mutants. We hypothesize that the explanation for the increased sensitivity displayed by the overexpression mutants could relate to iron metabolism, as the toxicity of phleomycin is directly correlated with intracellular iron contents (Haas *et al.*, 1999). Therefore, it might be that *atrB* is also involved in iron uptake or secretion of siderophores.

A better understanding of the role of AtrBp in sensitivity and resistance to toxicants may elucidate additional functions of AtrBp. This is of general relevance, since it might help to design strategies to overcome MDR in practice. This is already exemplified by our observation that dithiocarbamate fungicides and other compounds showed increased activity against overexpression mutants of *atrB*, with an MDR phenotype.

EXPERIMENTAL PROCEDURES

Strains, plasmids, and media

The *A. nidulans* strains and plasmids used in this study are listed in Table 3. All strains were derived from Glasgow stocks. Standard techniques for manipulation and growth were as described by Pontecorvo *et al.* (1953). *E. coli* DH5α was used as a host in plasmid propagation.

Nucleic acid manipulations and molecular biological techniques

Freshly harvested conidia obtained from confluent plate cultures of *A. nidulans*, grown for 4-5 days at 37 ^{0}C, were used as inoculum source for liquid cultures at a density of 10^7 conidia ml^{-1}. Germlings harvested after 14 hours of incubation at 37 ^{0}C were used for nucleic acid isolation according to Raeder & Broda (1985) and Logemann *et al.* (1987). The Random Primers DNA Labelling System (GIBCO BRL^{TM}) was used to generate radioactively labeled oligonucleotide probes with [α-^{32}P]dATP. Southern, Northern and dot blot hybridizations were performed using HybondN$^+$ (DNA) and HybondN (RNA) nylon membranes (Amersham), according to manufacturer's instructions. Overnight hybridized blots were washed at 65 ^{0}C with 0.1% SSC + 0.1% SDS solution. The pGEM-3Zf(+) and pGEM-T (Promega) vectors were used for cloning DNA fragments and PCR

products, respectively. Sequencing was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977). PCR reactions were performed using a Perkin-Elmer DNA thermal cycler 480 and Expand™ High Fidelity PCR kit (Boehringer Mannheim GmbH). Sequences were analyzed using the DNAstar package (DNASTAR).

Table 3. *Aspergillus nidulans* strains and plasmids used in this study.

a. Strains	**Genotype***	
WG488	*biA1; pyrG89; lysB5; fwA1; uaY9*	
PAO-1 and PAO-2	Independent monosporic transformants of WG488 with plasmid pAO4-2. Prototrophic for uridine.	
DB5 and DB21	WG488 with a single-copy replacement of *atrB* by the disruption construct DB. Independent monosporic transformants.	
PPL6-1	Monosporic transformants of WG488 with plasmid pPL6. Prototrophic for uridine.	
OB7, OB16 and OB35	Independent monosporic transformants of WG488 with plasmid pOB. Prototrophic for uridine.	
b. Plasmids	**Relevant characteristics†**	**Reference or source**
PGEM-3Zf$^{(+)}$	*E. coli* cloning vector	Promega
pGEM-T	*E. coli* cloning vector	Promega
pD15	Subclone containing *atrB* gene-specific probe	Del Sorbo *et al.*, 1997
pSF5	*gamma-actin* of *A. nidulans* cloned in pUC19	Fidel *et al.*, 1988
pGspD	Subclone containing *atrD* gene-specific probe	Andrade *et al.*, in press
pAO4-2	*pyrG* of *A. oryzae* cloned in pUC19	De Ruiter-Jacobs *et al.*, 1989
pPL6	*pyrG* of *A. nidulans* cloned in pUC19	Oakley *et al.*, 1987
pTB	*atrB* cloned in pGEM-T	This study
pAOB	*pyrG* of *A. oryzae* cloned in *Bam*HI site of pTB	This study
pOB	*atrB* cloned in *Eco*RI site of pPL6	This study

* For explanation of symbols, see Clutterbuck (1993).
† See methods for detailed information on cloning procedures.

Disruption constructs

Primers for amplification of the *atrB* locus were designed in the 5' and 3' UTR (untranslated regions). Artificial *Eco*RI sites were included in the primers to allow further subcloning of the PCR product. Primer sequences were 5'-CGTGAATTCCTGGATGGTTCAGCTTA-3' and 5'-TAAGAATTCTTCAAGTTCGTCGAAGACG-3'. A 5.2 kb amplified PCR product using the lambda clone an2 (Del Sorbo *et al.*, 1997) as template DNA, was cloned in pGEM-T and coded pTB. This clone was checked by restriction analysis and sequencing. Furthermore, the 8.0 kb pTB clone was restricted with

*Bam*HI and a 5.15 kb DNA fragment was used to clone the *pyrG* from *A. oryzae* as a 3.8 kb *Bam*HI insert from pAO4-2 restricted with *Bam*HI (De Ruiter-Jacobs *et al.*, 1989). This construct was coded pAOB. The final transformation construct, a 5.95 kb *Eco*RI DNA fragment (DB), was obtained by restriction of pAOB with *Eco*RI. For generation of the control strains, the pAO4-2 clone was used for transformation (De Ruiter-Jacobs *et al.*, 1989).

Overexpression constructs

The overexpression construct was made by restriction of pTB with *Eco*RI and a 5.2 kb DNA fragment containing the whole *atrB* locus was cloned in the *Eco*RI site of pPL6 (Oakley *et al.*, 1987). The resulting 9.4 kb vector, coded pOB, was used for transformation. The control strains (PPL6) were obtained by transformation with the pPL6 vector.

Preparation of protoplasts and transformation

Mycelial protoplasts were prepared as described by (Wernars *et al.*, 1985) with minor modifications. Liquid minimal medium supplemented with 2 g casamino acids l^{-1}, 0.5 g yeast extract l^{-1} and auxotrophic markers was inoculated with 10^6 conidia ml^{-1} and incubated overnight at 37 ^{0}C and 300 r.p.m. in a orbital incubator for 16 hours. The germlings were harvested through Mira-Cloth, washed twice with sterile water and twice with STC buffer (1.0 M sorbitol, 10 mM Tris-Cl pH 7.5, 50 mM $CaCl_2$) and squeezed between paper towels to remove excess of liquid. Protoplasts were released by incubation of one gram of mycelium at 30 ^{0}C and 100 r.p.m., resuspended in 20 ml of filter-sterilized iso-osmotic S0.8MC medium containing lytic enzymes (5 mg Novozym 234 ml^{-1}, 0.8 M KCl, 50 mM $CaCl_2$, 20 mM MES pH 5.8) for about 2 hours. The protoplast suspension was filtered over glass-wool, diluted (1:1, v/v) with STC buffer and incubated on ice for 10 min. Then, protoplasts were collected by centrifugation (10 min, 0 ^{0}C, 3000 r.p.m.) and washed twice with STC buffer. Transformation was performed as described by Van Heemst *et al.* (1997) using purified DNA of transformation constructs DB (3.5 µg) and pOB (5.0 µg) dissolved in sterile water (15 µl).

Toxicity assays

Sensitivity of *A. nidulans* strains to toxicants was determined by measuring their EC_{50} values for inhibition of radial growth on MM plates (De Waard and Van Nistelrooy, 1979). Mycelial agar plugs of an overnight-grown confluent plate of each strain were placed upside down on minimal medium (MM) plates amended with fungicides at different concentration of the compounds. Radial growth was assessed after 3 days incubation, at 37 ^{0}C. Carbendazim and sulfomethurom methyl were kindly provided by DuPont De Nemours, cilofungin by Eli Lilly, fenpiclonil, fludioxonil and trifloxystrobin by Novartis, kresoxim-methyl by BASF, fenarimol by Dow Elanco and imazalil nitrate and ketoconazole by Janssen Pharmaceuticals. All other chemicals tested were purchased from Sigma Chemical. For statistical analysis a radial growth test was performed in four replicates, at one concentration around the determined EC_{50} value of the compounds for the control strains. These concentrations were: azoxystrobin (0.05 μg ml^{-1}), carbendazim (0.3 μg ml^{-1}), cycloheximide (50 μg ml^{-1}), cyprodinil (0.03 μg ml^{-1}), eugenol (100 μg ml^{-1}), fenarimol (3 μg ml^{-1}), fenpiclonil (0.3 μg ml^{-1}), fluazinam (0.3 μg ml^{-1}), fludioxonil (0.1 μg ml^{-1}), imazalil nitrate (0.05 μg ml^{-1}), iprodione (5 μg ml^{-1}), itraconazole (0.05 μg ml^{-1}), kresoxim-methyl (0.05 μg ml^{-1}), miconazole (0.5 μg ml^{-1}), nigericin (3 μg ml^{-1}), Na-o-phenylphenate (15 μg ml^{-1}), nystatin (10 μg ml^{-1}), 4-nitroquinoline-*N*-oxide (1 μg ml^{-1}), phleomycin (30 μg ml^{-1}), prochloraz (0.1 μg ml^{-1}), propiconazole (1 μg ml^{-1}), pyrimethanil (0.3 μg ml^{-1}), quintozene (10 μg ml^{-1}), resveratrol (300 μg ml^{-1}), rhodamine 6G (5 μg ml^{-1}), thiabendazole (3 μg ml^{-1}), trifloxystrobin (0.01 μg ml^{-1}). The compounds were added from concentrated solutions in methanol. Amphotericin B (30 μg ml^{-1}), camptothecin (10 μg ml^{-1}), cilofungin (0.03 μg ml^{-1}), chlorothalonil (3.0 μg ml^{-1}), ferbam (30 μg ml^{-1}), and thiram (30 μg ml^{-1}), were added from concentrated solutions in DMSO. Acriflavine (3 μg ml^{-1}) was dissolved in sterile water. The final concentration of the solvents in the agar was the same for all treatments and never exceeded 1%. Analysis of variance from two independent experiments was applied as described by Snedecor and Cochran (1989). Significant differences were obtained by comparing the mean values of colony size of control strains and mutants using Tukey's test ($P<0.05$).

Accumulation of [^{14}C]fenarimol

Experiments were performed with standard suspensions of germlings of *A. nidulans* at an initial external concentration of 30 μM [^{14}C]fenarimol, as described before (De Waard and Van Nistelrooy, 1980).

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Chapter 5

ABC transporters and resistance to azole fungicides in the *ima* mutants of *Aspergillus nidulans* - a regulatory role for *imaB*

SUMMARY

We have determined the expression pattern of eight different ABC-transporter (Atr) encoding genes from *Aspergillus nidulans* that display high primary sequence homology to multidrug transporter proteins from other organisms. Transcription of all eight *atr* genes was studied in wild-type and multidrug resistant MDR (*ima*) mutants. Five (*atrA-D* and *atrC*$_2$) have been characterized previously and three *atr* candidate genes were selected from the expressed sequence tags (EST) database of *A. nidulans*. Selection of the EST clones was based on sequence homology to ABC proteins known to transport azoles. They were designated *atrE-G* and the full cDNA sequence of *atrE* is presented. *imaB* mutants of *A. nidulans* possess an increased energy-dependent efflux mechanism that prevents intracellular accumulation of fungicides and results in multidrug resistance to a range of compounds among which azole fungicides. The *imaB* mutation enhances transcription of several *atr* genes and this strongly suggests a mutation in a regulatory gene. Increased fenarimol efflux and decreased sensitivity to azole fungicides is also observed for an *atrB* overexpression mutant (OB35). However, fenarimol accumulation in *imaB* mutants is significantly lower than in OB35 indicating that the increased fenarimol efflux activity in *imaB* mutants is probably a concerted action of several overproduced Atr proteins, which may be AtrD-G.

INTRODUCTION

Antifungal sterol biosynthesis inhibitors are widely used in crop protection and clinical medicine. Major classes of sterol biosynthesis inhibitors are azoles (imidazoles and triazoles) and azole-related compounds (pyridines and pyrimidines). Azoles are site-specific inhibitors of either cytochrome P450-dependent lanosterol (yeasts) or eburicol (filamentous fungi) 14α-demethylase activity (Vanden Bossche, 1995). Inhibition of sterol demethylase activity results in depletion of ergosterol and accumulation of toxic sterol synthesis intermediates. Both effects cause malfunctioning of cell membranes and arrest of fungal growth. Fungicides with a specific mode of action can have a high risk of resistance development. This also applies to azole fungicides. Since their introduction, various cases of resistance development in either plant or mammalian pathogens have been reported (Denning *et al.*, 1997; Knight *et al.*, 1997; Vanden Bossche *et al.*, 1998; White *et al.*, 1998).

The major mechanisms of azole resistance in fungi are decreased affinity of the target enzyme sterol demethylase for the compounds and reduced accumulation of the compounds in the fungus (De Waard, 1994; White *et al.*, 1998). Resistance in mutants of *Aspergillus nidulans* selected under laboratory conditions for resistance to azole fungicides is due to decreased accumulation of these compounds. These mutants have a multidrug resistance (MDR) phenotype (Van Tuyl, 1977). MDR is the term used to describe the ability of cells to display cross-resistance to structurally unrelated compounds, after being selected for resistance to a single cytotoxic drug. This phenomenon has been reported to also operate in malignant cancer cells (Ambudkar *et al.*, 1999) and in a broad range of organisms, including causal agents of human diseases and is, therefore, of major concern in clinical medicine. The reduced accumulation of azoles in MDR mutants of *A. nidulans* is based on an increased energy-dependent efflux mechanism. (De Waard and Van Nistelrooy, 1979, 1980). This mechanism also operates in plant pathogens such as *Penicilium italicum*, *Botrytis cinerea*, *Nectria haematoccoca*, and probably *Mycosphaeaerella graminicola* (De Waard *et al.*, 1996; Joseph-Horne *et al.*, 1996). Also in the human pathogen *Candida albicans*, a similar mechanism has been reported (Ryley, 1984). In most instances, the increased energy-dependent efflux is due to overexpression of multidrug-efflux pumps, which drive the transport of the toxicants from the plasma membranes to the outer environment.

The major drug efflux pumps identified in microorganisms with affinity to azole antifungals belong to the **A**TP-**B**inding **C**assette (ABC) and the **M**ajor **F**acilator (MFS) superfamilies of proteins. In yeasts, examples of MFS transporters involved in resistance to azoles are *FLR1* from *Saccharomyces cerevisiae* (Alarco *et al.*, 1997) and *CaMDR1* from *C. albicans* (Fling *et al.*, 1991), respectively. The ABC family of proteins represents the largest class of transporters known to date (Bauer *et al.*, 1999). Besides multidrug transporters, the family includes proteins involved in membrane translocation of various substances such as ions, amino acids, peptides, sugars, vitamins, steroid hormones, bile acids and phospholipids (Higgins, 1992, 1994; Van Helvoort *et al.*, 1996). Analysis of the complete genome sequence of *S. cerevisiae* revealed 29 ABC-transporter encoding genes of which at least three (*PDR5*, *SNQ2* and *YOR1*) have been charcaterized as azole efflux-pumps. (Decottignies and Goffeau, 1997; Kolaczkowski *et al.*, 1998). In *C. albicans*, Cdr1p and Cdr2p are ABC transporters involved in resistance to azoles (Prasad *et al.*, 1995; Sanglard *et al.*, 1996, 1997). Overexpression of these drug efflux pumps in *S. cerevisiae* and *C. albicans* results in MDR indicating that these proteins have a broad

substrate specificity, accepting not only azoles but also structurally unrelated compounds as substrates.

To date, at least five ABC transporters from *A. nidulans* highly homologous to multidrug-efflux pumps from other organisms have been described. Additional sequences displaying homology to ABC transporters are present in an expressed sequence tag (EST) database (Andrade *et al.*, 1999; Angermayr *et al.*, 1999). In this report, we have analyzed the expression of *atrA-D*, ABC-transporter genes previously described (Andrade *et al.*, 1999; Angermayr *et al.*, 1999; Del Sorbo *et al.*, 1997) and the isolation of *atrE-G*, three new ABC-transporter genes. The expression was studied in a wild-type isolate and azole resistant mutants of *A. nidulans* with a MDR phenotype. The mutants, selected for resistance to the azole fungicide imazalil, carry *imaA*, *imaB* and *imaA* + *imaB* mutations (Van Tuyl, 1977). The single mutants *imaA* and *imaB* are isogenic strains derived from strain 003 but the recombinant strain 264 is not. *imaB* mutants display an increased energy-dependent efflux of the azole-like fungicide fenarimol. *atrD-G* show increased expression in strains carrying the *imaB* mutation as compared to wild-type and *imaA* strains. These results show that the *imaB* mutation affects transcription of several *atr* genes, suggesting that *imaB* may encode a transcriptional regulator. Results also suggest that AtrD-G proteins play a role in increased fenarimol-efflux activity of these mutants.

RESULTS

[^{14}C]fenarimol accumulation by azole resistant mutants

imaA and *imaB* mutants of *A. nidulans* are laboratory-generated mutants selected for resistance to the azole fungicide imazalil. They show cross-resistance to other azoles and azole-like compounds. Only the *imaB* mutants display decreased sensitivity to fenarimol (Fig. 1). In the *imaB* mutant, resistance to fenarimol is based on increased energy-dependent efflux activity which results in decreased cytoplasmic drug accumulation (De Waard and Van Nistelrooy, 1979, 1980). These results were confirmed (Fig. 2A). In addition, we found that [^{14}C]fenarimol accumulation by the *imaA* mutant, resembled that of the wild-type, being transient in time (Fig 2A). The non-isogenic recombinant strain 264 carrying both *imaA* and *imaB* mutations, showed a slightly higher degree of resistance and a increased accumulation of fenarimol as compared to strain 146 (Fig 1 and 2A).

Pre-treatment of germlings of wild-type isolate 003 with the protein synthesis inhibitor cycloheximide (200 μg ml^{-1}) for 60 min, inhibits the transient [^{14}C]fenarimol

accumulation pattern, suggesting that *de novo* protein synthesis is required for [^{14}C]fenarimol efflux (Fig. 2B). In contrast, pre-treatment with resveratrol (50 μg ml^{-1}) or fenarimol (20 μg ml^{-1}) decreases fenarimol accumulation, suggesting that these compounds induce the efflux activity (Fig. 2B).

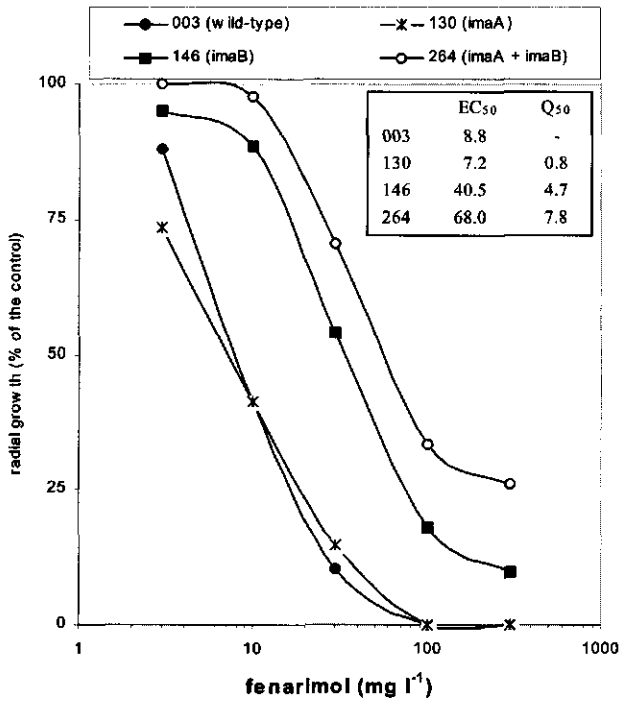


Figure 1. Sensitivity of wild-type and azole-resistant mutants of *Aspergillus nidulans* to fenarimol

Dose response curve indicating differential sensitivity of wild-type strain 003 (●) and azole-resistant mutants 130-*imaA* (*), 146-*imaB* (■) and 264-[*imaA* + *imaB*] (○) to fenarimol. The effective concentration required for 50% growth inhibition (EC_{50}) and the degree of sensitivity expressed as EC_{50} mutant/EC_{50} control strain (Q_{50}) are presented within figure.

Recently, we have demonstrated that the *atrB* overexpression mutant OB35 displays decreased sensitivity to fenarimol and other azole fungicides. [^{14}C]fenarimol accumulation by the *atrB* overexpression mutant is relatively low and energy-dependent. These results suggest that AtrBp accepts these fungicides as substrates (Andrade *et al.*, 2000). However, the relationship, between AtrBp and the *imaB* mutation is not clear. A comparison of [^{14}C]fenarimol accumulation by the *imaB* mutant 146 and the *atrB* overexpression mutant OB35 in relation to their parental

isolates (003 and PPL6, respectively) indicates that mutant OB35 shows a lower initial [^{14}C]fenarimol accumulation than isolate PPL6 (Fig. 2C). This is probably due to constitutive production of AtrBp. Accumulation of [^{14}C]fenarimol by the *imaB* mutant 146 is significantly lower, suggesting that the fenarimol efflux activity(ies) of the *imaB* mutant might be the result of additional fenarimol transporters different of AtrBp (Fig. 2C).

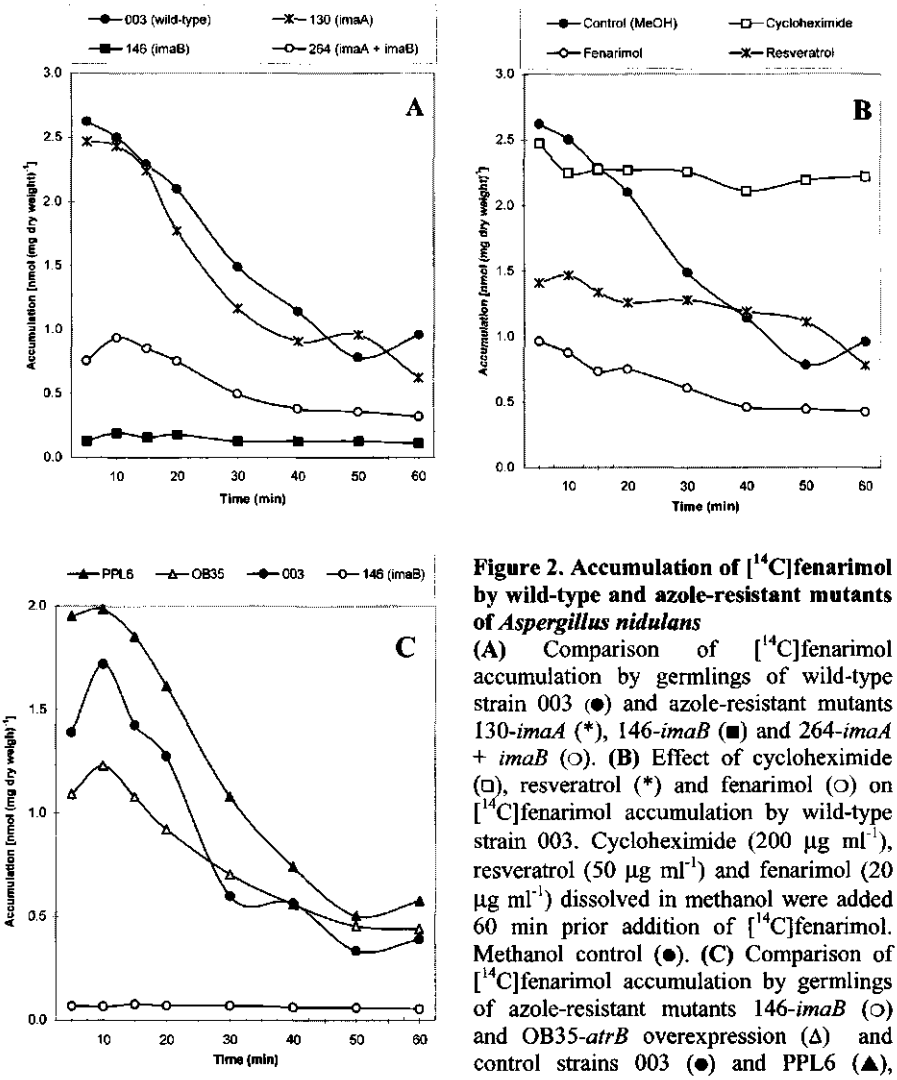


Figure 2. Accumulation of [^{14}C]fenarimol by wild-type and azole-resistant mutants of *Aspergillus nidulans*

(A) Comparison of [^{14}C]fenarimol accumulation by germlings of wild-type strain 003 (●) and azole-resistant mutants 130-*imaA* (*), 146-*imaB* (■) and 264-*imaA* + *imaB* (○). **(B)** Effect of cycloheximide (□), resveratrol (*) and fenarimol (○) on [^{14}C]fenarimol accumulation by wild-type strain 003. Cycloheximide (200 µg ml^{-1}), resveratrol (50 µg ml^{-1}) and fenarimol (20 µg ml^{-1}) dissolved in methanol were added 60 min prior addition of [^{14}C]fenarimol. Methanol control (●). **(C)** Comparison of [^{14}C]fenarimol accumulation by germlings of azole-resistant mutants 146-*imaB* (○) and OB35-*atrB* overexpression (Δ) and control strains 003 (●) and PPL6 (▲), respectively.

Generation of *atrA* knock-out mutants

Previous work demonstrated that *atrA* shows a higher basal transcript level in *imaB* mutants than in the wild-type isolate (Del Sorbo *et al.*, 1997), suggesting that the ABC transporter *atrA* can account for the high [^{14}C]fenarimol efflux observed in *imaB* mutants. To verify this hypothesis, we have generated a knock-out mutant of *atrA* by replacing the major part of its coding region by the orotidine-5'-phosphate decarboxylase (*pyrG*) encoding-gene of *Aspergillus oryzae*. The uridine-auxotrophic strain WG488 of *A. nidulans* was used as the recipient strain for transformation and selection of transformants was based on uridine prototrophy. A schematic representation of the disruption strategy used is presented in Fig. (3A). Southern blot analysis of genomic DNA isolated from 48 uridine prototrophic transformants revealed that in four transformants, a replacement of the *atrA* locus by the disruption construct DA had occurred (data not shown). However, in only one mutant, coded DA1, a single integration of the construct DA had occurred. The three other disruption mutants carried additional ectopic integrations of the disruption construct. Southern blot analysis with genomic DNA isolated from mutant DA1 confirmed replacement of the wild-type allele of *atrA* by the disruption construct DA (Fig. 3B). To confirm that *atrA* was functionaly deleted, Northern blot analysis was carried out with total RNA isolated from germlings of the control strains and *atrA* deletion mutants, treated with cycloheximide, a previously identified inducer of *atrA* transcription (Del Sorbo *et al.*, 1997). Surprisingly, for all strains tested, no detectable signal could be observed in the autoradiographs. The experiment was repeated three times with the same results. The activity of the probe was checked by hybridization with a Southern blot and results confirmed it to be functional.

To test the involvement of AtrAp in transport of azoles, a radial growth test was used to assess the sensitivity of the *atrA* knock-out mutants to azole fungicides. Sensitivity was compared with two control strains coded PAO-1 and PAO-2, obtained by transformation of strain WG488 with a contruct containing only the *pyrG* from *A. oryzae*. In this test, the *atrA* deletion mutant DA2 which has one additional ectopic integration of the disruption construct, was also included. The deletion mutants grew normally and no differences in radial growth rates as compared to the control stains were observed. Sensitivity of the *atrA* deletion mutants DA1 and DA2 to azole fungicides (fenarimol, imazalil, prochloraz, propiconazole and miconazole) was similar to the control strains (data not shown). These results indicate that AtrAp is not a transporter of azole fungicides and that other transporters are responsible for fenarimol-efflux activity.

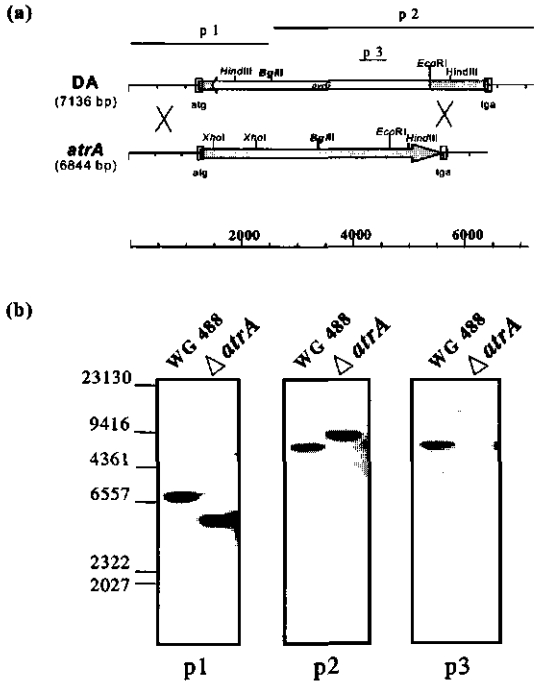


Figure 3. Replacement of the *atrA* gene of *Aspergillus nidulans*
(A) Schematic representation of the disruption construct, and wild-type and knock-out locus of *atrA*. Lines labeled p1, p2 and p3 indicate the restriction fragments used as probes in Southern blot analysis. **(B)** Southern blot analysis performed with DNA of the recipient strain WG488 used for transformation and the *atrA* deletion mutant DA1. Genomic DNA of WG488 and DA1 was restricted with *Bgl*II and hybridized with probes p1(left panel), p2 (middle panel) and p3 (right panel).

Characterization of ESTs homologous to azole ABC transporters from other fungi

The presence of sequences in an EST database of *A. nidulans* (Roe *et al.*, 1998) homologous to ABC transporters, have been reported previously (Andrade *et al.*, 1999; Angermayr *et al.*, 1999). In order to identify additional ABC genes from *A. nidulans* with a putative role in fenarimol efflux by *imaB* mutans, we studied EST sequences with homology to previously characterized azole transporters from other fungi. A TBLASTn search using the amino acid sequences of the azole transporters Pmr1p from *P. digitatum* (Nakaune *et al.*, 1998), Pdr5p from *S. cerevisae* (Balzi *et al.*, 1994) and Cdr1p from *C. albicans* (Prasad *et al.*, 1995) against the dBEST database of the NCBI site (http://www.ncbi.nlm.nih.gov/blast/blast.cgi) yielded a number of EST fragments displaying homology to different parts of the query protein sequence. Redundancy of

EST clones was observed and contigs based on the consensus of these redundant EST sequences were generated (Table 1 and Fig. 4). An alignment of the putative peptides encoded by these EST sequences with the amino acid sequence of Pmr1p is presented in Figure 4.

Since contigs 1, 2 and 3 displayed homology to the same C-terminal half of the query protein sequence, at least three new ABC transporters were identified. EST sequences displaying homology to the N-terminal half of the query protein sequence and contigs with homology to the C-terminal half could be parts of the same ABC transporter. To test this hypothesis we have followed a PCR approach using specific EST primers in different combinations. Using a cDNA library from *A. nidulans* as a template, PCR products of the expected size as compared to the Pmr1p sequence, were obtained in reactions using primer combinations *PF4* (specific primer for EST g0g01a1.f1) and *PR3* (contig 1) , and *PF3* (specifc primer for EST f0d04a1.r1) and *PR4* (contig 2) (Fig 4). The PCR products generated were coded PCR1 and PCR2, respectively. Products were also obtained when the primer combinations *PF1* (EST g0g01a1.r1) and *PR1* (EST g0g01a1.f1) coded PCR3, and *PF5* (EST 05g05a1.r1) and *PR5* (contig 3) coded PCR4, were used. These last combinations (*PF1* + *PR1*; *PF5* + *PR5*) were used as positive controls, since they were designed based on sequences (forward- .r1, and reverse- .f1) coming from the same EST clone (Roe *et al.*, 1998). All PCR products were fully sequenced. Overlapping sequences from PCR products PCR1 and PCR3, and the known sequences of contig 1 and clone g0g01a1 (Fig. 4) resulted in a full-length sequenced cDNA, which was coded *atrE*. The resulting consensus sequences obtained from PCR products PCR2 and contig 2 was coded *atrF*, and the resulting consensus sequences from PCR4 and contig 3, coded *atrG*. These latter sequences (*atrF* and *atrG*) correspond to partial ABC-transporter sequences. An unrooted phylogenetic tree that illustrates the relationship of several fungal ABC-transporter proteins sharing the [NBF-TM_6] configuration is presented in Fig. (5A). The tree is based on an alignment of the C-terminal transmembrane domain (TM_6-TM_{12}) of proteins from different fungal species (Fig. 5B). This region was chosen due to the lack of N-terminal sequences of *atrF* and *atrG*. The tree indicates that the identified ABC transporters *atrE-G* have high similarity to Pmr1p, a well characterized azole ABC transporter from *P. digitatum* (Nakaune *et al.*, 1998).

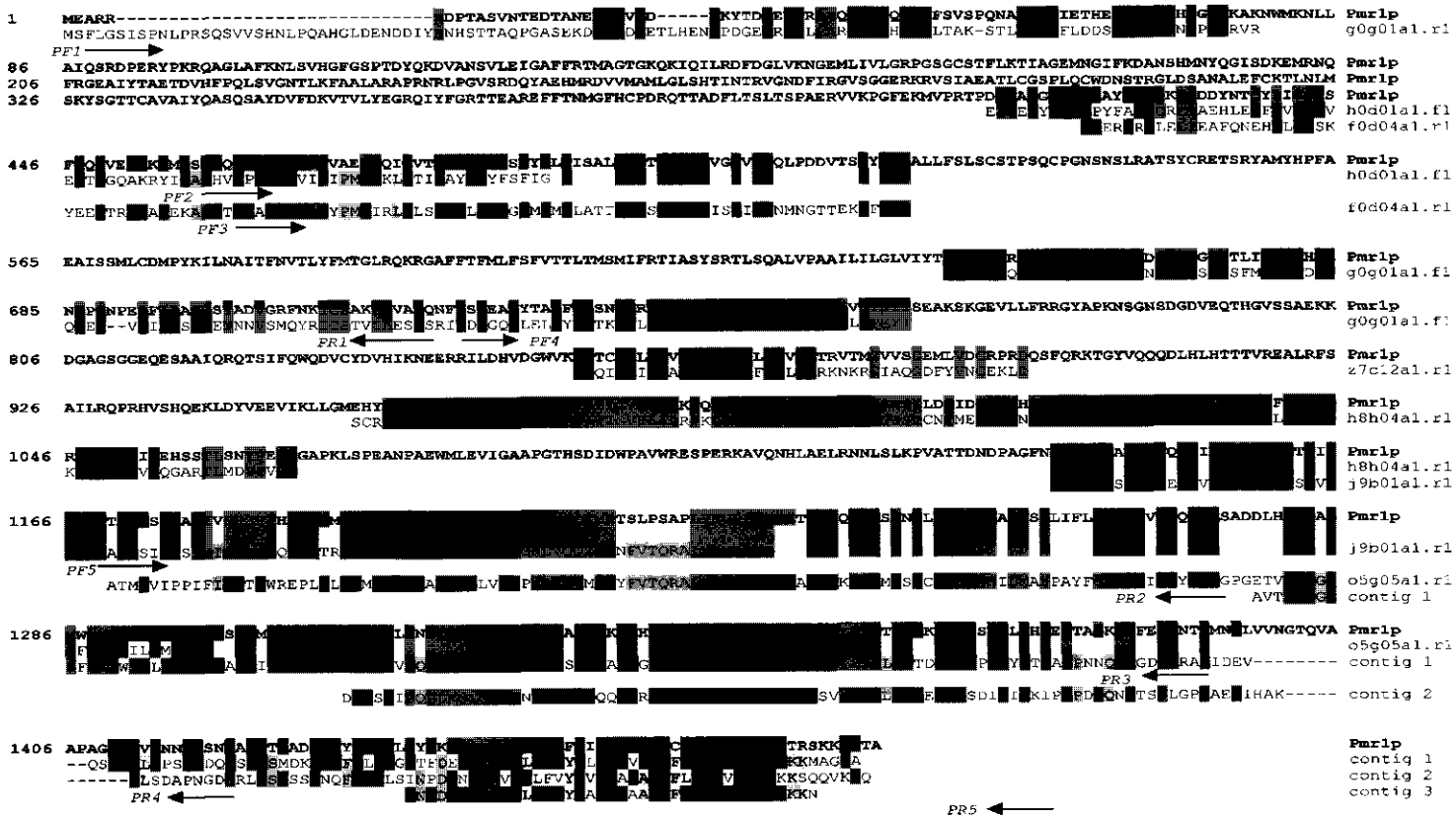


Figure 4. Alignment Amino acid alignment of Pmr1p with encoded peptides from different EST sequences and contigs derived from redundant clones, detected in the dBEST database at NCBI. Dark grey background indicates identical residues. Conservative substitutions are highlighted by a light grey background. Sequences were aligned using the Clustal W program (Thompson, 1994). Arrows indicate the position and orientation of olignucleotide primers designed for each EST clone.

Table 1. Redundant EST clones of *Aspergillus nidulans* encoding putative ABC proteins, detected using the amino acid sequence of Pmr1p[a] from *Penicillium digitatum* as the query sequence.

Consensus sequence	EST clones	GenBank number
Contig 1.	s3f01a1.f1	AA966005
	s3g01a1.f1	AA966007
	y8g05a1.f1	AI213250
	y8g05a1.r1	AI213251
	z6a05a1.r1	AI213841
Contig 2.	c8ea2a1.r1	AA783851
	c3a08a1.r1	AA783193
	c8e02a1.f1	AA783850
	n0c02a1.f1	AI211053
Contig 3.	g3c12a1.r1	AA784913
	j9b01a1.r1	AA786256
	k5e11a1.r1	AA965611
	xlf03a1.r1	AI212281

[a] Genbank accession number BAA31254.

(a)

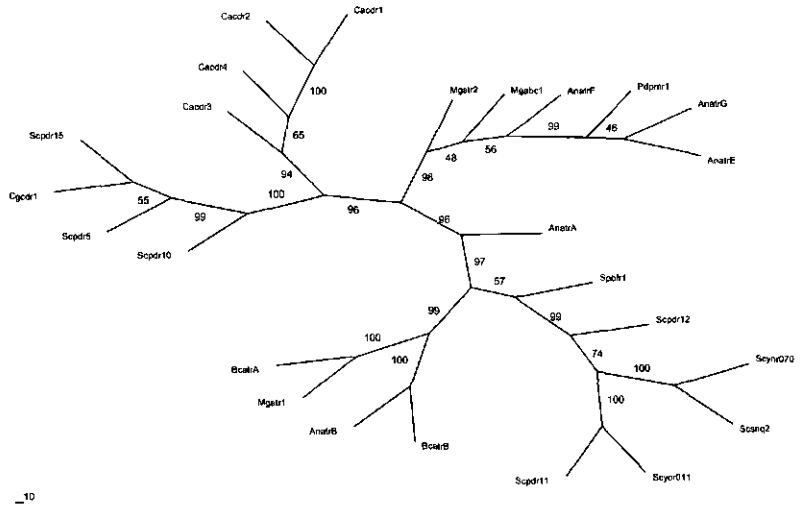
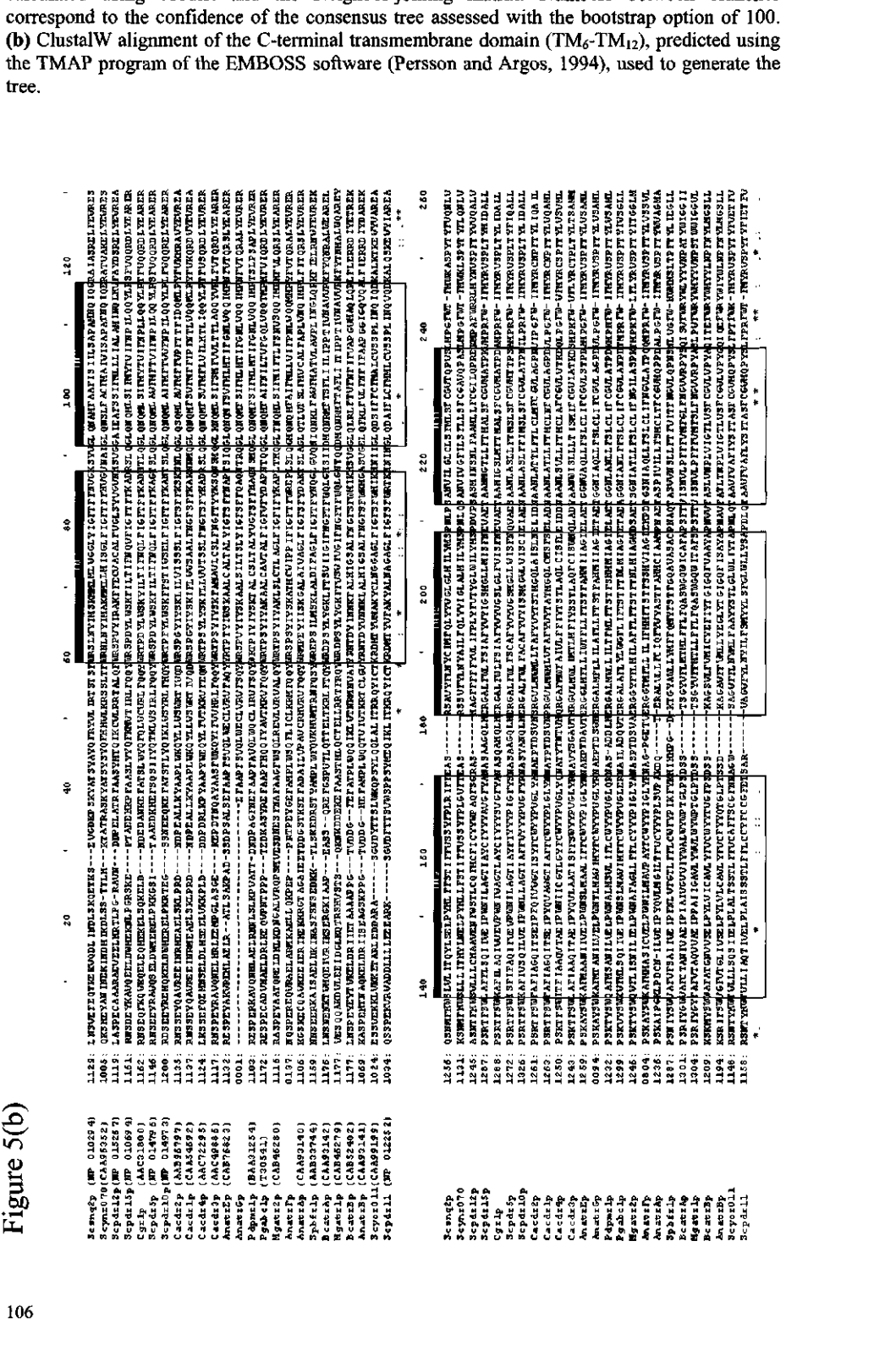


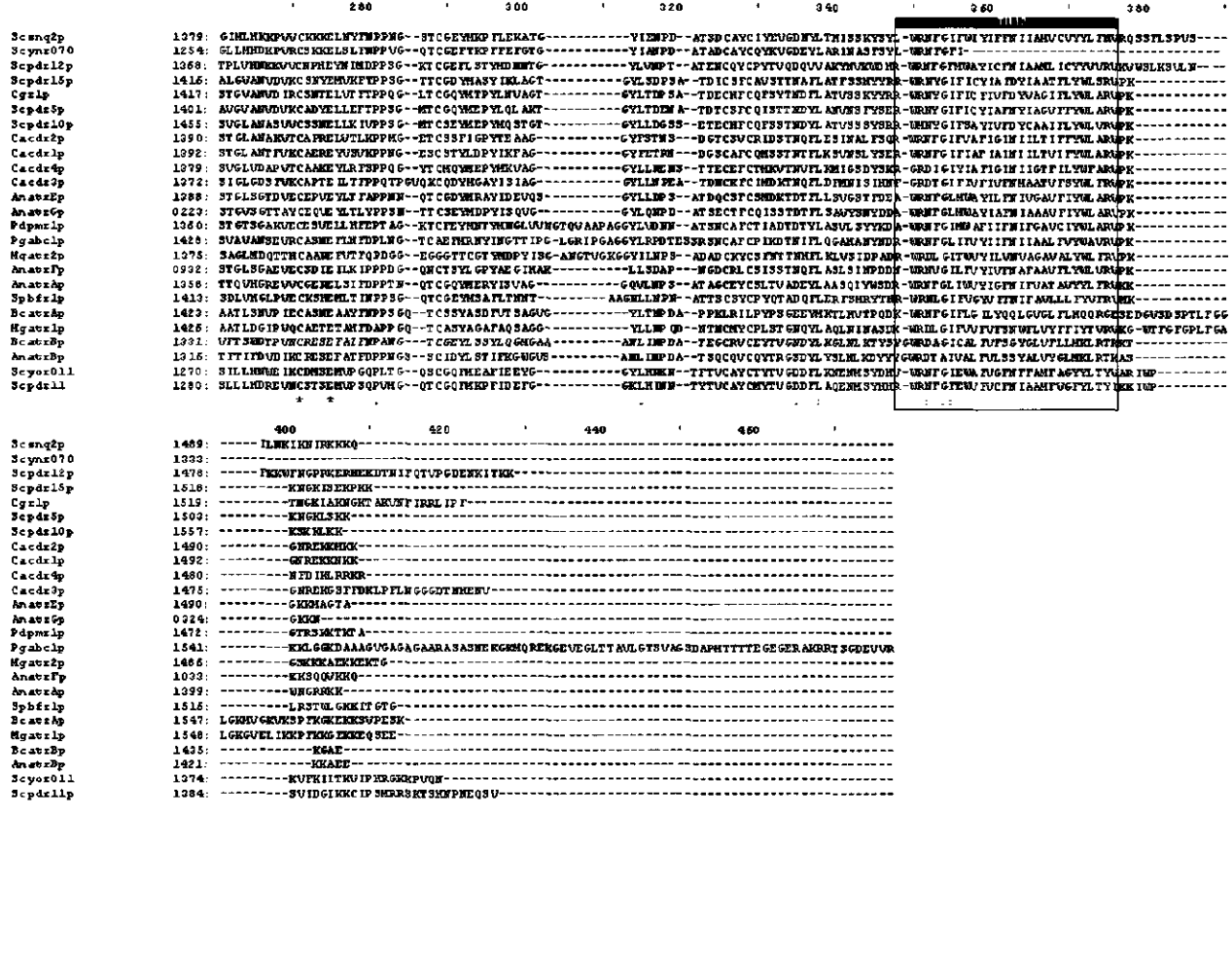
Figure 5. Phylogenetic tree

(a) Unrooted phylogenetic tree of several fungal ABC transporters with the $[NBF\text{-}TM_6]_2$ configuration. The consensus tree made with the Phylip program is presented. Distances were

calculated using Prodist and the Neighbor-joining matrix. Numbers between branches correspond to the confidence of the consensus tree assessed with the bootstrap option of 100. **(b)** ClustalW alignment of the C-terminal transmembrane domain (TM_6-TM_{12}), predicted using the TMAP program of the EMBOSS software (Persson and Argos, 1994), used to generate the tree.

Figure 5(b)





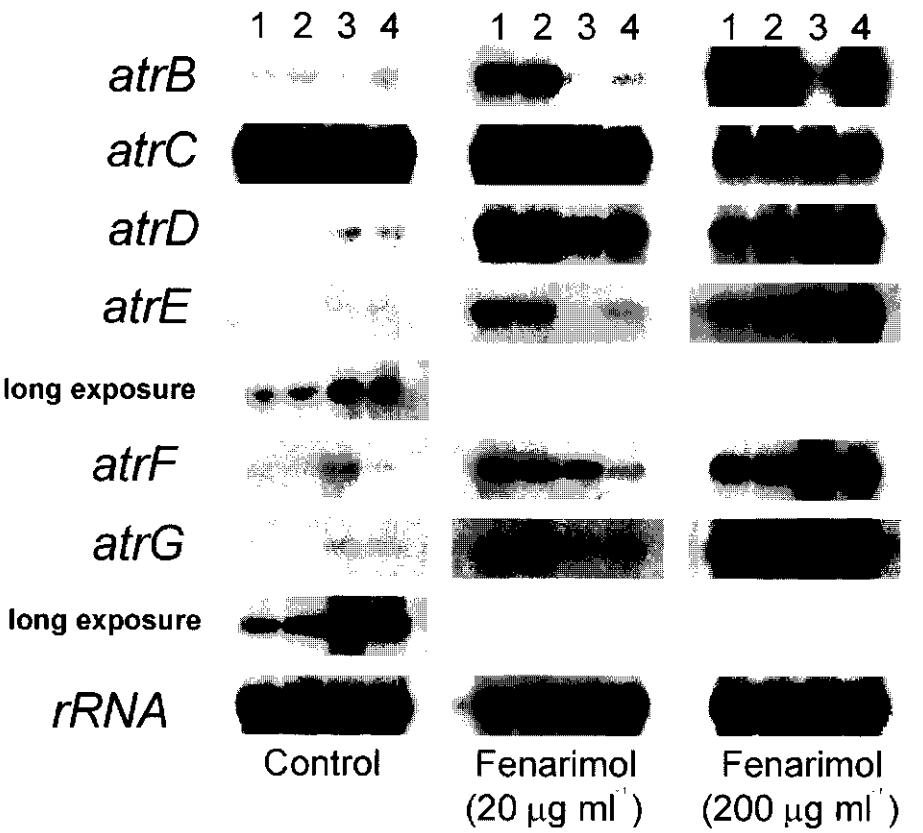


Figure 6. Northern Blot analysis
Northern blot analysis of *atrB-G* (top to bottom, respectively), using total RNA from germlings of wild-type and *ima* mutants of *Aspergillus nidulans*, treated with fenarimol for 60 min. Left panel, mock-treatment (methanol 0.1%), middle panel (fenarimol 20 µg ml^{-1}) and right panel (fenarimol 200 µg ml^{-1}). Wild-type 003 (lanes 1), strain 130-*imaA* (lanes 2), strain 146-*imaB* (lanes 3) and strain 264-*imaA* + *imaB* (lanes 4). Equal loading of samples was checked by hybridization with a ribosomal probe of *Aspergillus niger* (Melchers *et al.*, 1994) (Bottom pannel).

Expression analysis of *atr* genes in *ima* mutants

Transcription of *atr* genes was investigated by Northern blot analysis of total RNA isolated from four different strains (003, 130, 146 and 264). Total RNA was isolated from germlings treated with fenarimol (20 µg ml^{-1} and 200 µg ml^{-1}) or solvent control (methanol 0.1 %), for 60 minutes. For *atrA* and *atrC2*, no mRNA signal could be observed in any of the treatments (data not shown). Northern Blot analysis of the other *atr* genes showed a relatively high basal level of *atrC* expression as compared to all other genes tested (Fig. 6). The basal expression pattern of *atrC* is similar in all isolates

tested. *imaB* mutants show a higher basal level of expression of *atrD-G*. Treatment of fungal germlings with fenarimol (20 μg ml^{-1}) enhanced transcription of *atrB-G*, in all strains, but the effect is much less pronounced in the *imaB* mutant 146. Treatment with fenarimol at a higher concentration (200 μg ml^{-1}) resulted in higher *atrD-G* transcript levels in *imaB* mutants, as compared to the other strains tested but had no effect on *atrB* transcript levels in the *imaB* mutant 146 (Fig. 6). The treatment also enhanced *atrB* transcript levels in strains 003 and 130.

Treatment with resveratrol (50 μg ml^{-1}) specifically enhanced *atrB* transcript levels. Results indicate again that basal transcription of *atrB* is similar for all isolates tested (Fig. 7, left panel). The phytoalexin resveratrol increased transcription of *atrB* in all isolates tested (Fig. 7, right panel), but stronger in *imaB* mutants as compared to the wild-type 003 and *imaA* mutant 130. These results suggest a regulatory role of *imaB* on *atrB* transcription. This treatment also resulted in a negative effect on *atrE* transcript levels (data not show). In summary, Northern analysis indicate that *imaB* mutants possess a significant change in expression patterns of *atr* genes as compared to the wild-type 003.

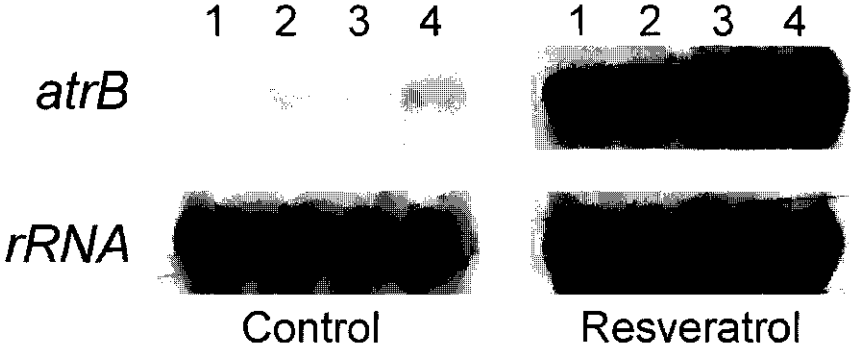


Figure 3. Expression of *atrB* upon treatment with resveratrol
Northern blot analysis of *atrB* (top panel) using total RNA from germlings of *Aspergillus nidulans* mock-treated with 0.1% methanol (left panel) and resveratrol (50 μg ml^{-1}) treated (right panel) for 60 min. Wild-type 003 (lanes 1), strain 130-*imaA* (lanes 2), strain 146-*imaB* (lanes 3) and strain 264-*imaA* + *imaB* (lanes 4). Equal loading of samples was checked by hybridization with a ribosomal probe of *Aspergillus niger* (Bottom panel).

DISCUSSION

Data presented in this paper provide molecular evidence for the fact that resistance to fenarimol in *imaB* mutants of *A. nidulans* is based on an increased energy-dependent

efflux mechanism that prevents intracellular accumulation of the fungicide (De Waard and Van Nistelrooy, 1980). Increased energy-dependent efflux commonly results from overproduction of multidrug-efflux pumps of either the MFS or ABC superfamily of proteins. For a better understanding of the role of ABC transporters in fenarimol-resistant *imaB* mutants of *A. nidulans*, we have determined the expression patterns of eight different *atr* genes that display high sequence homology to multidrug transport proteins from other organisms. Results indicate that *imaB* affects transcription of several *atr* genes simultaneously and therefore, it is unlikely that it encodes a structural transporter itself. We hypothesize that *imaB* is a mutation in a regulatory gene of *A. nidulans* that controls the expression of several structural genes. In that context, *imaB* could function in a similar way as *PDR1* of *S. cerevisiae* (Balzi *et al.*, 1987). Pdr1p is part of the PDR (pleitropic drug resistance) network of *S. cerevisiae* and functions as a transcriptional regulator of several ABC transporters as well as other transcription factors (Balzi and Goffeau, 1995).

Pre-treatment of wild-type *A. nidulans* germlings with cycloheximide (a protein synthesis inhibitor) annulled the transient accumulation curve of fenarimol indicating inhibition of fenarimol efflux. This result demonstrates that in the wild-type strain, fenarimol efflux requires *de novo* protein synthesis. In contrast, pre-treatment with fenarimol and resveratrol resulted in lower accumulation of fenarimol, indicating induction on the fenarimol efflux mechanism. Resveratrol specifically enhances transcription of *atrB*, especially in *imaB* mutants. These results suggest that resveratrol and fenarimol induce expression of *atrB* resulting in enhanced fenarimol efflux activity and reduced fenarimol accumulation. This is in agreement with our findings that indeed AtrBp is a multidrug transporter which also transport resveratrol and azole fungicides (Andrade *et al.*, 2000). Accumulation levels of fenarimol by *atrB* overexpression mutant OB35 lies between those of the wild-type isolate (003) and *imaB* mutant (146), indicating a higher efflux activity in *imaB* mutants as compared to *atrB* overexpression mutants. This result suggest a higher efflux activity in the *imaB* mutant than in the atrB overexpression mutant and may be indicative for a role of additional transporters in fenarimol efflux. This hypothesis is in agreement with previous results (Andrade *et al.*, 2000).

We have tested the role of additional ABC transporters in efflux of fenarimol by studying the transcription of various ABC genes (*atrA-G*) in wild-type and *ima* mutants of *A. nidulans*. AtrAp could have been a good candidate since basal transcript levels of *atrA* were reported to be higher in *imaB* mutants than in wild-type (Del Sorbo *et al.*,

1997). However, this study demonstrates that sensitivity of wild-type and *atrA* deletion mutants to azole fungicides is the same. We were also unable to reproduce the differential expression of *atrA* in wild-type and *imaB* mutants after pre-treatment with fungicides such as fenarimol (results not shown). Hence, we conclude that AtrAp is not a transporter of azole fungicides.

ABC transporter genes different from *atrA-D* were identified in an EST database of *A. nidulans* (Roe *et al.*, 1998). Translated sequences with high homology to well characterized azole transporters from other fungi (Balzi *et al.*, 1994; Nakaune *et al.*, 1998; Sanglard *et al.*, 1995) were selected. At least three different ABC transporters highly homologous to azole efflux pumps were identified. The cDNA of one of them, *atrE*, was fully and of the other two, *atrF* and *atrG*, were only partially sequenced. Phylogenetic analysis indicates that all three genes have high homology to Pmr1p, an ABC transporter from *P. digitatum* (Nakaune *et al.*, 1998). Resistance to azole fungicides in *P. digitatum* is correlated with enhanced expression levels of *Pmr1*. Treatment of mycelium with azole fungicides results in stronger *Pmr1* expression in azole-resistant than in wild-type isolates of *P. digitatum* (Nakaune *et al.*, 1998). These results indicate that Pmr1p plays a role in resistance to azole fungicides in *P. digitatum*.

We found that the ABC-transporter encoding genes *atrD*, *atrE*, *atrF* and *atrG* display a higher basal level of expression in *imaB* mutants than in wild-type *A. nidulans*. These results suggest that the higher fenarimol efflux activity in *imaB* mutants as compared to *atrB* overexpression mutants is the result of a concerted activity of multiple transporters. This situation resembles the PDR network of *S. cerevisiae* where several ABC transporters are co-regulated by Pdr1p (Balzi and Goffeau, 1995). Such a mechanism would also be in agreement with the hypothesis of Nakaune et al. (1998), that drug efflux transporters different from Pmr1p are involved in resistance to azole fungicides in *P. digitatum*. Our current research focuses on a further characterization of *atrF* and *atrG* and the isolation of the *imaB* gene.

EXPERIMENTAL PROCEDURES

Strains, plasmids, and media

All *A. nidulans* strains used in this study were derived from Glasgow stocks (Table 2). Standard techniques for manipulation and growth were as described by Pontecorvo *et al.* (1953). *E. coli* DH5α was used as a host in plasmid propagation.

Table 2. *Aspergillus nidulans* strains used in this study.

Strain	Genotype*
WG488	*biA1; pyrG89; lysB5; fwA1; uaY9*
PAO-1 and PAO-2	Independent monosporic transformants of WG488 with plasmid pAO4-2. Prototrophic for uridine.
DA1	WG488 with a single-copy replacement of *atrA* by the disruption construct DA.
DA2	WG488 with replacement of *atrA* by the disruption construct DA and one additional ectopic integration of the disruption contruct.

* For explanation of symbols, see Clutterbuck (1993).

Nucleic acids manipulations and molecular biological techniques

Freshly harvested conidia obtained from confluent plate cultures of *A. nidulans*, grown for 4-5 days at 37 ^{0}C, were used as inoculum source for liquid cultures at a density of 10^7 conidia ml^{-1} of medium. Germlings harvested after 14 hours of incubation at 37 ^{0}C were used for nucleic acid isolation according to Raeder & Broda (1985) and Logemann *et al.* (1987). Poly A$^+$ mRNA was purified from total RNA with the oligodex-dT^{TM} Qiagen kit (Qiagen, Chatsworth, CA, USA). The Random Primers DNA Labelling System (GIBCO BRL^{TM}) was used to generate radioactively labelled oligonucleotide probes with [α-^{32}P]dATP. Southern and Northern blot hybridizations were performed using HybondN$^+$ nylon membranes (Amersham), according to manufacturer's instructions. Overnight hybridised blots were washed at 65 ^{0}C with 0.1% SSC + 0.1% SDS solution. The pGEM-3Zf(+) and pGEM-T (Promega) vectors were used for cloning DNA fragments and PCR products, respectively. PCR reactions were performed using a Peltier Thermal Cycler PTC-200 (MJ Research) and Expand^{TM} High Fidelity PCR kit (Boehringer Mannheim GmbH) was used to amplify the disruption construct of *atrA*. Sequencing was carried out by the dideoxy chain-termination method (Sanger *et al.*, 1977). Sequences were analysed using the DNAstar package (DNASTAR).

The oligonucleotide primers used for amplification and sequencing the cDNA fragments of *atrE-F* are listed on Table 3. cDNA synthesis was performed using the Marathon^{TM} cDNA amplification kit with the Advantage® cDNA polymerase mix (Clontech, Palo Alto, Ca, USA). PCR conditions were as recommended by the the Marathon^{TM} cDNA amplification kit. Briefly, 94 ^{0}C for 1 min, 5 cycles (94 ^{0}C for 30 sec,

72 min for 4 min), 5 cycles (94 ^{0}C for 30 sec, 70 min for 4 min), 30 cycles (94 ^{0}C for 20 sec, 68 min for 4 min) and final extension at 68 ^{0}C for 6 min.

Table 3. Primers used to amplify and sequence *atrE*, *atrF* and *atrG*.

Code	Sequence 5'-3'
PF1	TTCTCGGATCCATCTCACCAAACC
PF2	AAGCATGTTCGCCCAAAAAGT
PF3	TGCTGCTTCACCTTACACA
PF4	GTGGACGGCGGACAATACCTG
PF5	GGCCGCACTGAGCATCCTG
PR1	TGACCCCGACTCTGCTCCAACTG
PR2	GACCGGCGTTGCGATAGAGC
PR3	TGTAGGCCCGCATATAGTCTCCA
PR4	GCAGTCACCATTAGGAGCATCAT
PR5	ACCCTCGCAACATTACGCAAAAA
AtrEf1	AGTGGTGAAATGCTTGTTGTCCTG
AtrEf2	GTCGACAGGAAAAGCTTGATTATG
AtrEf3	GCTTCGGCTCCCCAACTGATTAC
AtrEr1	AGAAGAGCACCCCGGTAGTAAAAA
AtrEr2	ATAGAGGTCGGGGCGTTGAAGAA
AtrEr3	CGCTCTGGGACGCTTGGTAG
AtrFf1	TATGCTTTTGAGTCCCTTATGGTC
AtrFr1	GGCGGGATTGGCATTCTTT
AtrFr2	CCCCTAGGATACCAACCACAGC

Disruption constructs

Primers for amplification of the *atrA* locus were designed in the 5' and 3' UTR (untranslated regions). Primer sequences were 5'-TCAATTCCCGCTCTGATCATCACAGG-3' and 5'-GGCACAATTCCAAGTGAACG -3'. A 6.7 kb amplified PCR product using the lambda clone an1 (Del Sorbo *et al.*, 1997) as template DNA, was cloned in pGEM-T and coded pTA. This clone was checked by restriction analysis and sequencing. Furthermore, the 9.7 kb pTA clone was restricted with *Eco*RI + *Xho*I and a 6.3 kb DNA fragment was used to clone the *pyrG* from *A. oryzae* as a 3.8 kb insert from pAO4-2 restricted with *Eco*RI + *Sal*I (De Ruiter-Jacobs *et al.*, 1989). This construct was coded pAOA. The final transformation construct, a 7.14 kb amplified product coded (DA) was obtained in a PCR reaction using the primers described above. For generation of the control strains, the pAO4-2 clone was used for transformation (De Ruiter-Jacobs *et al.*, 1989).

Preparation of protoplasts and transformation

Mycelial protoplasts were prepared as described by Wernars *et al.* (1985) with minor modifications. Liquid minimal medium supplemented with 2 g casamino acids l^{-1}, 0.5 g yeast extract l^{-1} and auxotrophic markers was inoculated with 10^6 conidia ml^{-1} and incubated overnight at 37 ^{0}C and 300 r.p.m. in an orbital incubator for 16 hours. The germlings were harvested through Mira-Cloth, washed twice with sterile water and twice with STC buffer (1.0 M sorbitol, 10 mM Tris-Cl pH 7.5, 50 mM $CaCl_2$) and squeezed between paper towels to remove excess of liquid. Protoplasts were released by incubation of one gram of mycelium, resuspended in 20 ml of filter-sterilized iso-osmotic S0.8MC medium containing lytic enzymes (5 mg Novozym 234 ml^{-1}, 0.8 M KCl, 50 mM $CaCl_2$, 20 mM MES pH 5.8) at 30 ^{0}C and 100 r.p.m. for about 2 hours. The protoplast suspension was filtered over glass-wool, diluted (1:1) with STC buffer and incubated on ice for 10 min. Then, protoplasts were collected by centrifugation (10 min, 0 ^{0}C, 3000 r.p.m.) and washed twice with STC buffer. Transformation was performed as described by Van Heemst *et al.* (1997) using purified DNA of transformation constructs DA (5 µg) dissolved in sterile water (15 µl).

Toxicity assays

Sensitivity of *A. nidulans* strains to toxic compounds was determined by measuring their EC_{50} values for inhibition of radial growth on MM plates (De Waard and Van Nistelrooy, 1979). Mycelial agar plugs of an overnight-grown confluent plate of each strain were placed upside down on a minimal medium (MM) plate containing different concentrations of the test compound. Radial growth was assessed after 3 days incubation at 37 ^{0}C.

Accumulation of [^{14}C]fenarimol

Experiments were performed with standard suspensions of germlings of *A. nidulans* at an initial external concentration of 30 µM [^{14}C]fenarimol, as described before (De Waard and Van Nistelrooy, 1980).

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Chapter 6

Sensitivity to phenylpyrrole fungicides in *Aspergillus nidulans* is mediated by the multidrug-efflux pump AtrB

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SUMMARY

The multidrug-efflux protein AtrBp from *Aspergillus nidulans* can act as a transporter of the phenylpyrrole fungicides fenpiclonil and fludioxonil and is a determinant of cellular sensitivity of this fungus to these compounds. Deletion and overexpression mutants of *atrB* have increased and decreased sensitivity to the fungicides, respectively, as compared to control strains. The sensitivity of the mutants to fludioxonil is positively correlated with accumulation levels of the fungicide in germlings. We also found that in overexpression mutants with different levels of *atrB* expression and in deletion mutants, transcript levels of *atrB* negatively correlates with sensitivity to fludioxonil and accumulation of the fungicide by germlings. In all isolates accumulation of fludioxonil was energy-dependent, indicating that reduced accumulation of the fungicide in the overexpression mutants is due to increased efflux activity of AtrBp. We propose that AtrBp functions as an energy-dependent efflux pump that modulates intracellular concentration of phenylpyrrole fungicides in fungal mycelium.

INTRODUCTION

Fungi, bacteria and higher plants are an important natural source of molecules with antifungal properties (Knight *et al.*, 1997). Phenylpyrrole fungicides are an example of antifungal compounds developed by modification of a natural toxic product. These fungicides were developed based on the lead structure pyrrolnitrin, a secondary metabolite of *Pseudomonas pyrocina* and other pseudomonads (Nyfeler and Ackermann, 1992). Pyrrolnitrin is effective in the control of several post-harvest diseases (Hammer *et al.*, 1993) but its use in agriculture is limited since the pyrrole ring is photounstable. Pyrrolnitrin also displays some phytotoxicity (Fischer *et al.*, 1992). Optimization of photostability led to the introduction of the highly active 3-cyanopyrroles fenpiclonil (Nevill *et al.*, 1988) and fludioxonil (Gehmann *et al.*, 1990). Fenpiclonil is used as a seed-dressing agent against numerous fungal pathogens. Fludioxonil shows improved photostability over fenpiclonil, and it has been exploited as a foliar fungicide against *Botrytis cinerea*, *Monilia* spp., and *Sclerotinia* spp. (Nyfeler and Ackermann, 1992).

The genetic potential of microorganisms to evolve resistance mechanisms in response to new fungicides can not be underestimated (Knight *et al.*, 1997). Several mechanisms of fungicide resistance have been identified and can relate to (i) enzymatic

inactivation or degradation of compounds, (ii) alterations of the drug target-site and, (iii) decreased intracellular accumulation of the toxic compounds. Many modern fungicides are single-site inhibitors of fungal metabolism and can have a high risk of resistance development due to alteration of the drug target site. Biochemical studies indicate that phenylpyrrole fungicides affect cell wall synthesis and induce accumulation of glycerol in mycelium (Jespers *et al.*, 1993; Leroux *et al.*, 1992; Pillonel and Meyer, 1997). Several lines of evidence suggest that their primary target site could be protein kinases involved in the regulation of polyol biosynthesis (Orth *et al.*, 1995; Pillonel and Meyer, 1997; Schumacher *et al.*, 1997). Under laboratory conditions, mutants from different fungal species displaying cross resistance to both phenylpyrrole and dicarboximide fungicides can readily be isolated. These resistant mutants also display hypersensitivity to osmotic stress and mutants from plant pathogens such as *Botrytis cinerea* and *Ustilago maydis*, are non-pathogenic (Beever, 1983; Ellis *et al.*, 1991; Faretra and Pollastro, 1993; Orth *et al.*, 1995). This phenotype of resistant mutants possibly accounts for the low frequency of these type of mutants under field conditions (Leroux *et al.*, 1999). Field isolates of *B. cinerea* which acquired resistance to dicarboximides after a few years of commercial use were not osmotically sensitive, suffered only from a minor fitness penalty and were sensitive to phenylpyrroles (Hilber, 1992).

Drug extrusion, mediated by membrane-associated drug efflux pumps is another ingenious mechanism used by microorganisms to evade the toxic effects of antibiotics (Putman *et al.*, 2000). The major drug-efflux pumps identified in microorganisms belong to the **A**TP-**B**inding **C**assette (ABC) and the **M**ajor **F**acilitator (MFS) superfamilies of proteins (Marger and Saier, 1993; Van Veen and Konings, 1998). Some of these drug-efflux pumps, the so-called multidrug transporters, have specificity for compounds with very different chemical structures and cellular targets. Overexpression of these energy-dependent multidrug efflux proteins is often associated with resistance to several chemically unrelated drugs, a biological phenomenon common to many organisms and termed multidrug resistance (MDR). In filamentous fungi, MDR was first reported for laboratory-generated mutants of *A. nidulans* selected for resistance to azole fungicides (De Waard and Van Nistelrooy, 1979; Van Tuyl, 1977). Resistance to azoles due to enhanced energy-dependent efflux of these compounds also operates in plant pathogens such as *Penicilium italicum*, *Botrytis cinerea*,

Nectria haematoccoca, and probably *Mycosphaeaerella graminicola* (De Waard *et al.*, 1996; Joseph-Horne *et al.*, 1996).

To date, several genes encoding ABC proteins from a number of filamentous fungi highly homologous to multidrug-efflux pumps have been described (Andrade *et al.*, 2000a, 1999; Angermayr *et al.*, 1999; Del Sorbo *et al.*, 1997; Nakaune *et al.*, 1998; Tobin *et al.*, 1997; Urban *et al.*, 1999; Zwiers and De Waard, 1999). *AtrA* and *atrB* from *A. nidulans* were the first ABC-transporter genes to be reported from a filamentous fungus (Del Sorbo *et al.*, 1997). AtrBp is a well characterized multidrug-efflux protein that confers MDR when overexpressed in *S. cerevisiae* or *A. nidulans* (Andrade *et al.*, 2000b; Del Sorbo *et al.*, 1997). Genes encoding proteins with very high homology to AtrBp have already been described for *B. cinerea* and *M. graminicola* (Goodall *et al.*, 1999; Schoonbeek *et al.*, 1999). This suggest that these proteins may have a similar function.

This paper reports that the multidrug-efflux protein AtrBp from *A. nidulans* functions as an energy-dependent efflux pump that modulates intracellular concentration of phenylpyrrole fungicides in fungal mycelium. Results show that AtrBp protects fungal cells against phenylpyrrole fungicides and can be a major determinant in resistance of this fungus to these fungicides

RESULTS

Sensitivity of deletion and overexpression mutants of *atrB* to phenylpyrrole fungicides

To characterize the role of AtrBp in protection of *A. nidulans* against phenylpyrrole fungicides, we tested the sensitivity of deletion and overexpression mutants of *atrB* to fludioxonil and fenpiclonil. These mutants have been described previously (Andrade *et al.*, 2000b). Briefly, deletion mutants were generated by replacing a major part of the coding region of *atrB* with the orotidine-5'-phosphate decarboxylase (*pyrG*) encoding-gene of *A. oryzae*, using a uridine-auxotrophic strain of *A. nidulans* (WG488) as the recipient strain for transformation. Generation of overexpression mutants of *atrB* was achieved by transformation of the same strain (WG488) with construct pOB that contains a genomic copy of *atrB* comprising the coding region plus the 5' and 3'untranslated regions (UTR). This contruct (pOB) was cloned in the pPL6 vector (Oakley *et al.*, 1987) which contains the *pyrG* from *A. nidulans*. Transformants of *A. nidulans* with different levels of increase in *atrB* expression were selected. Characterization of these transformants resulted in strains OB7, OB16 and OB35, with a

low, intermediate and high increase in basal expression level of *atrB*, respectively (Andrade *et al.*, 2000b).

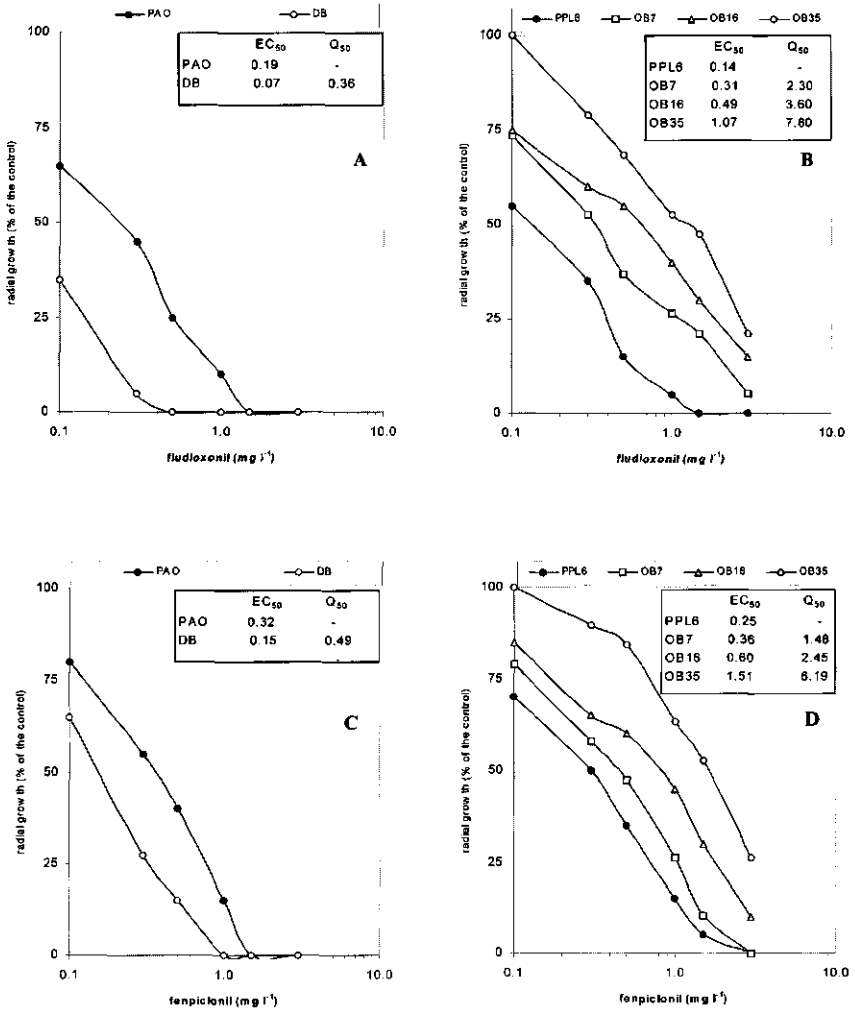

Figure 1. Effect of phenylpyrroles on radial growth of deletion and overexpression mutants of *atrB* from *Aspergillus nidulans*

Dose-reponse curve of control strains PAO (●) and *atrB* deletion mutants DB (○) to fludioxonil **(A)** and fenpiclonil **(C)**. Dose-reponse curve of control strain PPl6 (●) and *atrB* overexpression mutants OB7 (□), OB16 (△) and OB35 (●) to fludioxonil **(B)** and fenpiclonil **(D)**. Data for strains PAO and DB represent mean values of independent monosporic transformants PAO-1 and PAO-2, and DB5 and DB21, respectively. Results represent mean values of three repetitions. The effective concentration required for 50% growth inhibition (EC_{50}) and the degree of sensitivity expressed as EC_{50} mutant/EC_{50} control strain (Q_{50}) are presented within each panel.

Radial growth toxicity tests with fludioxonil and fenpiclonil indicates that deletion mutants of *atrB* (DB) display increased sensitivity to both phenylpyrrole fungicides tested, as compared to the control strains PAO (Fig 1A and 1C). In contrast, the overexpression mutants OB7, OB16 and OB35 display decreased sensitivity to both fungicides. Furthermore, the degree of resistance of these overexpression mutants was positively correlated with the levels of *atrB* expression (Fig 1B, 1D and 2).

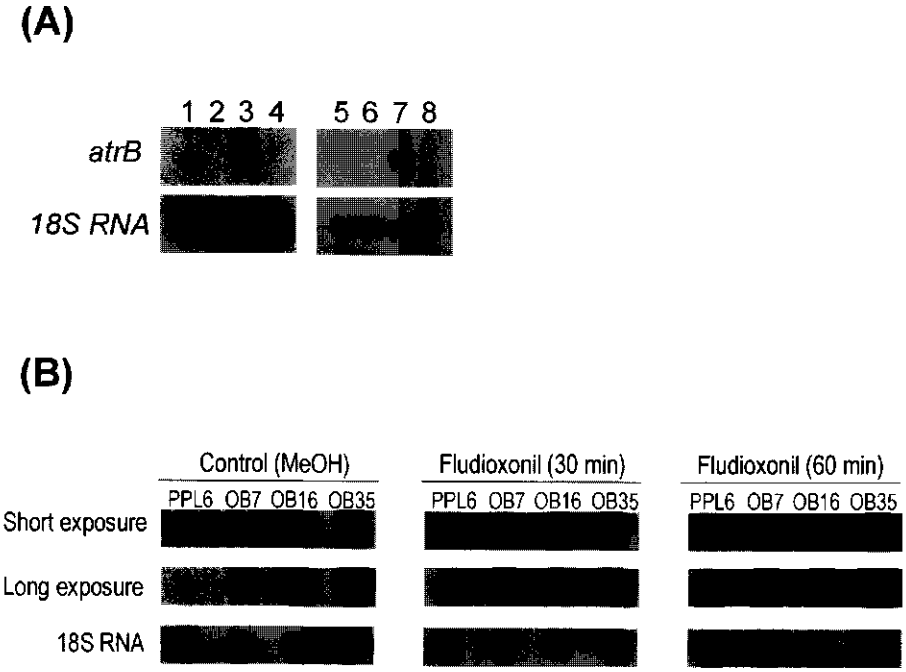

Figure 2. Northern blot analysis of *atrB* upon treatment of *Aspergillus nidulans* with fludioxonil

(A) Northern blot analysis of *atrB* (top panel) using total RNA from germlings treated with fludioxonil for 30 min (lanes 3 and 4) or 60 min (lanes 7 and 8). Methanol control, 0.1% (lanes 1, 2 and 5, 6). Control strain PAO-1 (lanes 1, 3, 5 and 7), *atrB* deletion mutant DB-5 (lanes 2, 4, 6 and 8). Equal loading of samples was checked by hybridization with a ribosomal probe of *Aspergillus niger* (bottom panel). **(B)** Northern blot analysis of *atrB* (top and middle panels) using total RNA from germlings of *atrB* overexpression mutants of *A. nidulans* treated with fludioxonil for 30 min (middle panel) or 60 min (right panel) after different exposure times of blots (short and long exposure). Methanol control, 0.1% (left panel). Equal loading of samples was checked by hybridization with a ribosomal probe of *Aspergillus niger* (bottom panel).

Expression analysis of *atrB* upon treatment with fludioxonil

To further characterize the role of *atrB* in sensitivity of *A. nidulans* to phenylpyrrole fungicides, we have investigated the effect of fludioxonil treatment on *atrB* expression. This was performed by Northern blot analysis of RNA isolated from fludioxonil-treated (4 μM) and mock-treated (0.1% methanol) germlings of the deletion and overexpression mutants of *atrB* and their corresponding control strains PAO and PPL6, respectively. Fludioxonil treatment strongly enhances *atrB* transcript levels in control strain PAO1, but no detectable signal for *atrB* is observed in total RNA isolated from fludioxonil-treated germlings of strain DB5 (Fig 2A). These results confirm functional deletion of *atrB* in strain DB5. Results also show that strains OB7, OB16 and OB35 have a higher basal level of *atrB* transcripts as compared to control strain PPL6 (Fig. 2B, left panel). These levels are different among OB strains and increase in the order of OB7, OB16 and OB35 (Fig. 2B, left panel). Fludioxonil treatment (30 or 60 min) strongly enhances *atrB* transcription in control strains PPL6 and in the overexpression mutants OB7 and OB16 (Fig 2A and 2B). Treatment of germlings from strain OB35 with fludioxonil does not obviously affect *atrB* transcript levels (Fig 2B).

[^{14}C]fludioxonil accumulation by *atrB* mutants

To demonstrate that AtrBp functions as an energy-dependent efflux pump that prevents intracellular accumulation of phenylpyrrole fungicides in germlings of *A. nidulans*, [^{14}C]fludioxonil accumulation by deletion and overexpression mutants of *atrB* was determined. *atrB* deletion strains (DB) display a higher [^{14}C]fludioxonil accumulation as compared to the control strains PAO (Fig 3A). Accumulation of [^{14}C]fludioxonil by control strains (PAO) is transient while that of the deletion strains is almost constant in time (Fig 3A). In contrast, *atrB* overexpression mutants OB7, OB16 and OB35 display lower [^{14}C]fludioxonil accumulation as compared to control strain PPL6 (Fig. 3B). Mutant OB35 shows the lowest [^{14}C]fludioxonil accumulation level of all strains tested while mutants OB16 and OB7 show intermediate accumulation levels. These results indicate that overexpression of *atrB* results in an increased efflux of [^{14}C]fludioxonil.

We also show that the accumulation of [^{14}C]fludioxonil in overexpression mutant OB35 and in control strain PPL6 is energy-dependent. This conclusion is based on the observation that the respiratory inhibitor CCCP instantly increased accumulation of [^{14}C]fludioxonil in both strains tested (Fig. 3C). This effect is ascribed to inhibition of

energy-dependent [^{14}C]fludioxonil efflux activity, in a similar way as described previously for [^{14}C]fenarimol (De Waard and Van Nistelrooy, 1980).

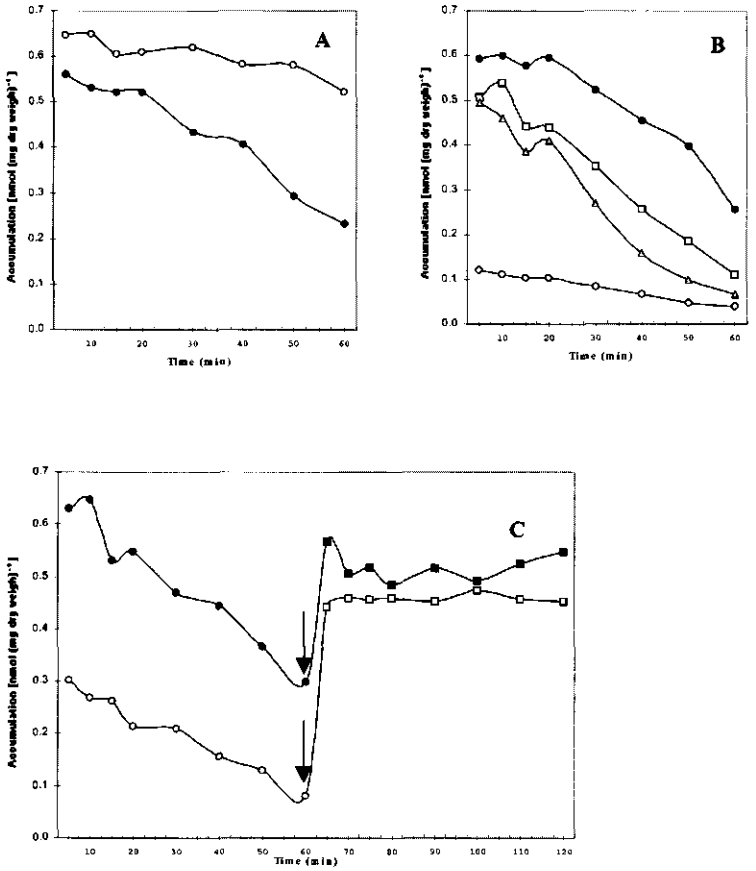

Figure 3. Accumulation of [^{14}C]fludioxonil by deletion and overexpression mutants of *atrB* from *Aspergillus nidulans*

(A) Comparison of [^{14}C]fludioxonil accumulation by germlings of control strains PAO (●) and *atrB* deletion mutants-DB (○). Data for strains PAO and DB, represents mean values of independent monosporic transformants PAO-1 and PAO-2, and DB5 and DB21, respectively. **(B)** Comparison of [^{14}C]fludioxonil accumulation by germlings of control strain PPL6 (●) and *atrB* overexpression mutants OB7 (□), OB16 (△) and OB35 (○). **(C)** Effect of CCCP (■,□) on [^{14}C]fludioxonil accumulation by control strain PPL6-1 (closed symbols) and the *atrB* overexpression mutant OB35 (open symbols). Arrows indicate CCCP (30 μM) added 60 min after addition of [^{14}C]fludioxonil (t=0).

DISCUSSION

The present study shows that AtrBp is a determinant of sensitivity of *A. nidulans* to phenylpyrrole fungicides. This conclusion is based on the observation that the expression level of *atrB* in deletion and overexpression mutants of *atrB* is negatively correlated with sensitivity to fenpiclonil and fludioxonil and, with accumulation levels of [^{14}C]fludioxonil in germlings.

AtrBp functions as an inducible, energy-dependent efflux pump of fludioxonil, since accumulation of [^{14}C]fludioxonil by control strains PAO and PPL6 is transient in time and addition of the respiratory inhibitor CCCP results in an instantaneous increase in accumulation of the fungicide. These results suggest that energy-dependent efflux activity of AtrBp prevents intracellular accumulation of phenylpyrrole fungicides, in a similar way as described for azole fungicides (De Waard and Van Nistelrooy, 1980, 1984).

Recently, we have reported that these overexpression mutants of *atrB* also accumulate relatively low levels of [^{14}C]fenarimol and are less sensitive to fenarimol (Andrade *et al.*, 2000b). These results indicate that AtrBp can also act as a fenarimol efflux pump. However, [^{14}C]fenarimol accumulation by deletion mutants of *atrB* was similar to control strains (Andrade *et al.*, 2000b). We hypothesized that *A. nidulans* has (an) additional efflux pump(s) accepting fenarimol as substrate that could compensate for the absence of AtrBp in *atrB* deletion mutants. In this paper, we demonstrate that deletion mutants of *atrB* display a relatively high levels of [^{14}C]fludioxonil accumulation and have an increased sensitivity to phenylpyrroles than control strains. Therefore, the ABC transporter AtrBp can be regarded as the major efflux pump of phenylpyrrole fungicides in *A. nidulans*.

Northern blot analysis indicates that treatment of fungal mycelium with fludioxonil results in an increase in *atrB* trancript levels and supports the involvement of AtrBp in protection against phenylpyrrole fungicides. However, this effect is not evident in the mutant OB35 which has a high basal level of *atrB* expression. Possibly, induction of *atrB* requires intracellular accumulation of fludioxonil, a condition which is not met in mutant OB35. Hence, the constitutively high efflux-activity of fludioxonil in mutant OB35 prevents that the fungicide reaches a sufficiently high concentration to enhance transcription of *atrB*.

The molecular basis for the broad substrate specificity of multidrug ABC proteins is only poorly understood (Gottesman *et al.*, 1995). However, recent studies

with the human P-glycoprotein (Loo and Clarke, 1999a, 1999b, 2000; Ueda *et al.*, 1997), the LmrAp from *Lactococcus lactis* (Van Veen *et al.*, 2000a, 2000b) and Pdr5p from *S. cerevisiae* (Egner *et al.*, 2000, 1998) have provided strong evidence that the transmembrane domains form the route through which the solutes cross the plasma membrane and confer substrate specifity by one or more substrate-binding sites. A BLAST analysis with the AtrBp sequence reveals that BcatrBp from *B. cinerea* is its closest homologue with an overall identity of 70%. A similar level of identity is also observed between AtrBp and Mgatr5p from *M. graminicola* (Goodall *et al.*, 1999). Most interestingly, the predicted transmembrane domains of both proteins are also highly conserved. This suggests that BcatrBp from *B. cinerea* and Mgatr5 from *M. graminicola* may have similar substrates as AtrBp from *A. nidulans*. The functional characterization of *BcatrB* from *B. cinerea* (Vermeulen *et al.*, 2000), indicates that *BcatrB* is indeed the *B. cinerea* orthologue of *atrB* from *A. nidulans*. We will compare the substrate specificity of AtrBp, BcatrBp and Mgatr5p by overexpression of their encoding genes in an *atrB* deletion mutant of *A. nidulans*. This type of experiments may validate *A. nidulans* as an alternative system to dissect the substrate specificity of ABC proteins from other filamentous fungi. This would be particularly helpfull for ABC transporters from human pathogens such as *Aspergillus fumigatus* and *Aspergillus flavus* where the generation of overexpression mutants of multidrug-efflux pumps in the laboratory requires extreme caution since they can be hazardous to human health.

EXPERIMENTAL PROCEDURES

Strains, plasmids, and media

The *A. nidulans* strains used in this study are listed in Table 1. All strains were derived from Glasgow stocks. Standard techniques for manipulation and growth were as described by Pontecorvo *et al.* (1953). *E. coli* DH5α was used as a host in plasmid propagation.

Nucleic acids manipulations and molecular biological techniques

Freshly harvested conidia obtained from confluent plate cultures of *A. nidulans*, grown for 4-5 days at 37 ^{0}C, were used as inoculum source for liquid cultures at a density of 10^{7} conidia ml^{-1}. Germlings harvested after 14 hours of incubation at 37 ^{0}C were used for nucleic acid isolation according to Logemann *et al.* (1987). The Random Primers DNA Labelling System (GIBCO BRL^{TM}) was used to generate radioactively labelled

oligonucleotide probes with [α-^{32}P]dATP. Northern blot hybridizations were performed using HybondN nylon membranes (Amersham), according to manufacturer's instructions. Overnight hybrydized blots were washed at 65 ^{0}C with 0.1% SSC + 0.1% SDS solution.

Table 1. *Aspergillus nidulans* strains and plasmids used in this study.

Strains	**Genotype***
WG488	*biA1; pyrG89; lysB5; fwA1; uaY9*
PAO-1 and PAO-2	Independent monosporic transformants of WG488 with plasmid pAO4-2. Prototrophic for uridine.
DB5 and DB21	WG488 with a single-copy replacement of *atrB* by the disruption construct DB. Independent monosporic transformants.
PPL6-1	Monosporic transformants of WG488 with plasmid pPL6. Prototrophic for uridine.
OB7, OB16 and OB35	Independent monosporic transformants of WG488 with plasmid pOB. Prototrophic for uridine.
Plasmids	**Relevant characteristics**
pD15	Subclone containing *atrB* gene-specific probe (Del Sorbo *et al.*, 1997)
p18S	Subclone containing a ribosomal probe from *Aspergillus niger* (Melchers *et al.*, 1994)

* For explanation of symbols, see Clutterbuck (1993). All strains are described in Andrade *et al.* (2000b).

Toxicity assays

Sensitivity of *A. nidulans* strains to the phenylpyrrole fungicides fludioxonil and fenpiclonil was determined by performing a dose-response curve for inhibition of radial growth on MM plates (De Waard and Van Nistelrooy, 1979). Mycelial agar plugs of an overnight-grown confluent plate of each strain were placed upside down on minimal medium (MM) plates amended with fungicides at different concentration. Radial growth was assessed after 3 days incubation at 37 ^{0}C. The final concentration of the solvents in the agar was the same for all treatments and never exceeded 1%.

Accumulation of [^{14}C]fludioxonil

Experiments were performed with standard suspensions of germlings of *A. nidulans* at an initial external concentration of 4 μM [^{14}C]fludioxonil using the same methodology described for experiments with [^{14}C]fenarimol (De Waard and Van Nistelrooy, 1980). Carbonyl cyanide *m*-chlorophenylhydrzone (CCCP) was purchased from Sigma-Aldrich.

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Chapter 7

General discussion

GENERAL DISCUSSION

Cytotoxic compounds such as drugs in clinical medicine and fungicides in control of plant diseases are widely used. However, the widespread and sometimes excessive use of these compounds has resulted in a high selection pressure resulting in drug resistant populations. Understanding the mechanisms of antimicrobial resistance is essential in order to cope with this serious problem.

In general, the major mechanisms underling resistance in microorganisms can be classified as follows: (a) enzymatic inactivation or degradation of drugs, (b) alterations of the drug target-site, and, (c) decreased drug-accumulation caused by energy-dependent drug efflux. More than one mechanism may operate in concert and the sum of different alterations represents the ultimate resistant phenotype.

Drug extrusion mediated by membrane-associated drug efflux pumps is an ingenious mechanism used by microoganisms to evade the toxic effects of antibiotics (Putman *et al.*, 2000). The major drug-efflux pumps identified in microorganisms belong to the **ATP-B**inding **C**assette (ABC) and the **M**ajor **F**acilitator (MFS) superfamilies of proteins (Marger and Saier, 1993; Van Veen and Konings, 1998). Some of these drug-efflux pumps, the so-called multidrug transporters, have specificity for compounds with very different chemical structures and cellular targets. Overexpression of these energy-dependent multidrug efflux proteins is often associated with resistance to several chemically unrelated drugs, a biological phenomenon common to many organisms and termed **multidrug** resistance (MDR).

The aim of this thesis was to identify and characterize molecular mechanisms for drug-resistance in *Aspergillus nidulans*, with special emphasis on drug-efflux systems of the ABC-transporter superfamily of proteins. Results presented in previous chapters and relevant aspects that may apply to other filamentous fungi are discussed here.

Isolation and characterization of *atr* genes from *Aspergillus nidulans*

Using different approaches, we have identified seven ABC-transporter genes (*atrA-G*) from *A. nidulans*. The approach of heterologous screening using a DNA probe from *PDR5*, a well characterized multidrug transporter from *Saccharomyces cerevisiae*, yielded the first ABC transporters, *atrA* and *atrB*, isolated from a filamentous fungus (Chapter 2). A second approach, based on PCR with degenerate oligonucleotide primers designed to amplify regions of the *A. nidulans* genome encoding consensus ABC-transporter sequences similar to P-glycoproteins, resulted in the cloning of *atrC* and

atrD (Chapter 3). The last approach was based on a screening of an EST database of *A. nidulans* for sequences encoding putative proteins with homology to known fungal multidrug transporters, particularly involved in efflux of azole fungicides. With this approach, *atrE*, *atrF* and *atrG* were identified (Chapter 5). The proteins encoded by all seven *atr* genes isolated display a high primary sequence homology to known multidrug-efflux proteins from other organisms. As described in Chapter 1, all members of the ABC-transporter superfamily have a modular architecture. The majority of the ABC transporters in higher organisms consists of two transmembrane domains (TMD), each with six predicted membrane spanning regions, and two intracellularly located nucleotide binding folds (NBF). The NBF domain can be either located at the N-terminus or at the C-terminus of the polypeptide, yielding proteins with a $[TMD\text{-}NBF]_2$ or $[NBF\text{-}TMD]_2$ configuration. The proteins encoded by *atrA* and *atrB* (Chapter 2) have the $[NBF\text{-}TMD]_2$ topology and are highly homologous to multidrug-transport proteins from yeasts (Balzi and Goffeau, 1994). The same configuration is displayed by AtrEp, AtrFp and AtrGp (Chapter 5). AtrCp and AtrDp (Chapter 3) have the mirror-like structure $[TMD\text{-}NBF]_2$, analogous to the human multidrug transporter P-glycoprotein (P-gp or MDR1) (Juranka *et al.*, 1989).

Expression analysis of *atr* genes

To further investigate the role of the isolated *atr* genes from *A. nidulans* in multidrug transport, expression analysis of these genes upon treatment with toxicants was performed with wild-type and MDR (*ima*) mutants of *A. nidulans*. *imaA* and *imaB* mutants of *A. nidulans* are laboratory-generated mutants selected for resistance to the azole fungicide imazalil and related compounds (Van Tuyl, 1977). In *imaB* mutants, resistance to fenarimol is based on an increased energy-dependent efflux activity that results in decreased cytoplasmic drug accumulation (De Waard and Van Nistelrooy, 1979, 1980). Therefore, these mutants were good candidates to use in our studies, to correlate azole resistance of these mutants with changes in expression of *atr* genes.

Expression studies in a wild-type isolate demonstrated that the basal level of *atr* gene expression is usually low and can be strongly enhanced by treatment with unrelated toxicants. Time course experiments, indicate that within 5 min after treatment with a toxicant (*e.g.* imazalil), enhanced transcript levels of *atr* genes can be observed (Chapter 2). These results are expected when one considers the energy-costs required for transport (low basal level) and a role in MDR (quick and broad response to

toxicants). Some compounds can have a specific effect on transcription of a particular *atr* gene while others may have an effect on transcript levels of several *atr* genes. The specific effect can be exemplified by results described in Chapter 5, where treatment with resveratrol specifically induces transcription of *atrB*. On the other hand, treatment with fenarimol enhances transcription of several (*atrB-G*) genes (Chapter 5). The latter observation suggests that some *atr* genes are co-regulated and the Atr proteins may have overlapping substrate specificities. This is in agreement with observations on ABC proteins from other organisms (Hirata *et al.*, 1994; Kolaczkowski *et al.*, 1998; Sanglard *et al.*, 1997).

Expression analysis in the *ima* mutants of *A. nidulans* shows that *atrD*, *atrE*, *atrF* and *atrG* display a higher basal level of expression in *imaB* mutants than in the wild-type (Chapter 5). These results suggest that increased fenarimol efflux activity in *imaB* mutants is the result of a concerted activity of multiple transporters and strongly suggest that *imaB* is a mutation in a regulatory gene. This situation resembles the PDR network of *S. cerevisiae* where several ABC transporters are co-regulated by Pdr1p (Balzi and Goffeau, 1995). Such a mechanism would also meet the assumption of Nakaune *et al.* (1998), that drug-efflux transporters different from Pmr1p are involved in resistance to azole fungicides in *Penicillium digitatum*.

Functional characterization of *atr* genes

atrB and *atrD* have been functionally characterized as multidrug transporters, since deletion mutants of these genes display increased sensitivity to a number of unrelated toxicants. Δ*atrB* mutants have an increased sensitivity to different classes of fungicides, mutagens and natural toxic compounds. On the other hand, Δ*atrD* mutants display increased sensitivity to cycloheximide, the cyclosporin derivative PSC 833, nigericin and valinomycin. These results indicate that AtrBp and AtrDp from *A. nidulans* are multidrug transporters with different substrate specifities (Chapters 3 and 4).

AtrBp was further characterized by overexpression in *A. nidulans* and *S. cerevisiae* (Chapters 2, 4 and 6). Sensitivity to cycloheximide of a *PDR5* deficient strain of *S. cerevisiae* was restored to wild-type levels, upon transformation with cDNA of *atrB* in a high copy number vector (Chapter 2). No changes in sensitivity to cycloheximide were observed for neither deletion nor overexpression mutants of *atrB* obtained by transformation in *A. nidulans*. Therefore, the substrate specificity of AtrBp

expressed in yeast and *A. nidulans* are not the same. It has been demonstrated for the human MDR1 protein that lipid composition of membranes can affect substrate specificity and ATPase activity (Doige *et al.*, 1993; Romsicki and Sharom, 1998; Sharom, 1997). Hence, differences in membrane composition of *S. cerevisiae* and *A. nidulans* could explain our results.

Overexpression mutants of *atrB* display altered sensitivity to a broader range of compounds as compared to Δ*atrB* mutants (Chapter 4), indicating that the presence of additional drug-efflux pumps with affinity for the same compound may prevent a change in phenotype of deletion mutants. Hence, the functional characterization of the substrate profile of a multidrug transporter using both deletion and overexpression mutants is particularly useful in order to overcome the problem of redundant transporters with similar specificities, in the same organism. Our studies indicate that fenarimol is an example of a compound that can be a substrate of various efflux-pumps in *A. nidulans*. Accumulation and drug-sensitivity assays indicate that overexpression mutants of *atrB* display relatively low levels of fenarimol accumulation and decreased sensitivity to fenarimol (Chapter 4). These results indicate that AtrBp can act as a fenarimol efflux pump. However, sensitivity of Δ*atrB* mutants to fenarimol and their fenarimol accumulation was similar to control strains, indicating that *A. nidulans* has (an) additional efflux pump(s) accepting fenarimol as substrate that can compensate for the absence of AtrBp in Δ*atrB* mutants (Chapter 4). Data from expression analyses also suggest the existence of several fenarimol-efflux pumps (Chapters 2, 3 and 5).

ABC transporters that transport similar compounds may also have distinct affinities for particular substrates. For instance, the functional characterization of *atrB* (Chapter 6) strongly supports that AtrBp has a distinctive affinity to fludioxonil since both Δ*atrB* and overexpression mutants have altered sensitivity to fludioxonil. In addition, changes in sensitivity observed for Δ*atrB* mutants correlate with the relatively high accumulation levels of fludioxonil. This was not observed for fenarimol. Therefore, the ABC transporter AtrBp can be regarded as a major efflux pump of phenylpyrrole fungicides in *A. nidulans*.

Redundance of ABC transporters may explain, at least in part, the findings that Δ*atrA* and Δ*atrC* mutants have no hypersensitive phenotype for any of the compounds tested. However, the observation that *atrA* transcript levels were not influenced by any of these compounds and that *atrA* expression in *S. cerevisiae* does not confer drug resistance,

suggest that AtrAp indeed is not a multidrug transporter. Alternatively, AtrAp can be a transporter of a non-toxic endogenous substrate, with an unknown physiological function.

Physiological functions of *atr* genes

The decreased antibiotic activity of *ΔatrD* mutants suggests that AtrDp might have a role in penicillin secretion (Chapter 3). In that case, AtrDp would be the first ABC transporter for which a role in secretion of a fungal antibiotic is demonstrated. The involvement of ABC-transporter proteins in secretion of the endogenous antibiotics (*e.g.* rubicin and mithramycin) has been demonstrated before for *Streptomyces peucetius* (Guilfoile and Hutchinson, 1991) and *S. argillaceus*, respectively (Fernandez *et al.*, 1996). The decrease in antibiotic activity of *ΔatrD* strains can be due to reduction of efflux capacity by AtrDp. However, alternative explanations are possible. AtrDp could also be involved in compartmentalization of biosynthetic precursors. In *A. nidulans*, the enzymes involved in penicillin biosynthesis are located in three different cellular compartments (Brakhage, 1998). Thus, in biosynthesis of penicillin several transport steps are required to bring intermediates of the penicillin biosynthesis pathway in contact with enzymes in the cytosol. If these transport steps would require AtrDp, disruption of the encoding gene would also affect penicillin production. Furthermore, AtrDp could have a yet unknown physiological function that could indirectly affect regulatory mechanisms of penicillin production. There is now accumulating evidence that the human multidrug transporter, MDR1, has several cellular functions and regulates a range of different physiological processes (Johnstone *et al.*, 2000). Similarly, AtrDp could have ion channel activity that affects extracellular pH, a well characterized regulatory-factor in penicillin biosynthesis of *A. nidulans* (Espeso *et al.*, 1993). Hence, further studies to support a physiological role of AtrDp in penicillin biosynthesis are needed. Generation of overexpression mutants of *atrD* and protein-localization studies would be helpful tools to address this question.

Interestingly, overexpression mutants of *atrB* displayed increased sensitivity to dithiocarbamate fungicides, chlorothalonil and the iron-activated antibiotic phleomycin (Chapter 4). This phenotype was most pronounced in the overexpression mutant with the highest levels of *atrB* expression. We hypothesize that this phenomenon could relate to iron metabolism. Phleomycin toxicity is directly correlated with intracellular concentration of iron (Haas *et al.*, 1999). Therefore, it might be that *atrB* is involved in iron uptake or secretion of siderophores. Preliminary expression studies (H. Haas,

personal communication) indicating higher transcript levels of *atrB* under iron limiting conditions, support this hypothesis.

Concluding remarks

The ABC superfamily of transporters comprises the largest protein family known to date. These proteins have substrate specifities ranging from ions, heavy metals, carbohydrates, drugs, amino acids, phospholipids, steroids, glucocorticoides, bile acids, mycotoxins, antibiotics, pigments, peptides and even large proteinaceous toxins (Bauer *et al.*, 1999).

This thesis demonstrates that some of the identified ABC transporters from *A. nidulans* can function in cellular protection against natural toxicants and xenobiotics. Deletion and overexpression mutants display increased and decreased sensitivity to toxicants, respectively. Data presented also suggest a role for ABC transporters in production of fungal secondary metabolites. This may imply that strains overexpressing multidrug-transporter genes can show various pleiotropic effects with respect to production of secondary metabolites. Such strains are of interest if they could increase production of commercially important compounds. However, they may pose a danger if they would also transport endogenous detrimental compounds such as microbial virulence factors or mycotoxins.

Genes encoding proteins with very high homology to AtrBp have recently been described for important plant pathogens such as *Botrytis cinerea* (*BcatrB*) and *Mycosphaerella graminicola* (*Mgatr5*) (Goodall *et al.*, 1999; Schoonbeek *et al.*, 1999). These ABC transporters may have similar substrates as AtrBp of *A. nidulans*. The functional characterization of *BcatrB* from *B. cinerea* (Vermeulen *et al.*, 2000) indicates that *BcatrB* from *B. cinerea* and *atrB* from *A. nidulans* are indeed orthologues. By comparing the substrate specificity of AtrBp, BcatrBp and Mgatr5p upon expression in *A. nidulans*, one can validate that *A. nidulans* is a suitable system to dissect the substrate specificity of ABC proteins from other filamentous fungi. This would be particularly helpful for ABC transporters from the human pathogens *Aspergillus fumigatus* and *Aspergillus flavus* where the generation of mutants overexpressing multidrug-efflux pumps requires extreme caution since these mutants can be extremely hazardous to human health.

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SUMMARY

The term multidrug resistance (MDR) stands for simultaneous cellular resistance to chemically unrelated toxicants and is often associated with overproduction of multidrug-efflux proteins of the ATP-binding-cassette (ABC) superfamily. The ABC transporters comprise a large and multifunctional family of proteins. Besides multidrug transporters, the superfamily includes proteins involved in transmembrane transport of various substances such as ions, amino acids, peptides, sugars, vitamins, steroid hormones, bile acids, and phospholipids. An overview of the great variety of cellular functions that these proteins can perform in living cells is presented in Chapter 1.

The aim of this thesis was to identify and characterize molecular mechanisms of drug resistance in *Aspergillus nidulans*, with special emphasis on drug-efflux proteins of the ABC-transporter superfamily. Using different approaches, we have identified seven ABC-transporter genes (*atrA-G*) from *A. nidulans*. Heterologous screening of a genomic library from *A. nidulans* using a DNA probe from *PDR5*, a well characterized multidrug transporter from *Saccharomyces cerevisiae*, yielded *atrA* and *atrB*, the first ABC-transporter genes isolated from a filamentous fungus (Chapter 2). The second approach, PCR with degenerate oligonucleotide primers based upon consensus sequences encoding ABC transporters from the subfamily of P-glycoproteins, resulted in the cloning of *atrC* and *atrD* (Chapter 3). The last approach, was based on a screening of an EST database of *A. nidulans* for sequences encoding proteins with homology to known fungal multidrug transporters, particularly involved in efflux of azole fungicides. With this approach, *atrE*, *atrF* and *atrG* were identified (Chapter 5). The proteins encoded by all seven *atr* genes isolated display high sequence homology to known multidrug-efflux proteins from other organisms.

To investigate the role of the isolated *atr* genes from *A. nidulans* in multidrug transport, expression analysis of these genes in wild-type and MDR (*ima*) mutants of *A. nidulans* was performed, after treatment of germlings with toxicants. *imaA* and *imaB* are laboratory-generated mutants of *A. nidulans* selected for resistance to the azole fungicide imazalil and related compounds. In *imaB* mutants, resistance to the azole-like compound fenarimol is based on an increased energy-dependent efflux activity which results in decreased cytoplasmic drug accumulation. Therefore, these mutants were suitable to correlate azole resistance with expression levels of *atr* genes.

Expression studies in a wild-type isolate demonstrated that the basal level of expression for most *atr* genes is low and can be strongly enhanced by treatment with unrelated toxicants (Chapters 2, 3, 5 and 6). Time course experiments indicated that within 5 min after treatment with a toxicant (*e.g.* imazalil), enhanced transcript levels of *atr* genes can be observed (Chapter 2). Some compounds can specifically induce transcription of one particular *atr* gene while others may simultaneously affect transcription of several *atr* genes (Chapter 5). For instance, resveratrol specifically induces transcription of *atrB*, while treatment with fenarimol enhances transcription of several genes (*atrB-G*). Expression analyses in the *ima* mutants of *A. nidulans* shows that *atrD*, *atrE*, *atrF*, and *atrG* display a higher basal level of expression in *imaB* mutants than in the wild-type (Chapter 5). Treatment with fenarimol also enhances transcription of these *atr* genes in *imaB* mutants.

Mutants in which *atrB* and *atrD* have been deleted display increased sensitivity to a number of unrelated toxicants. Δ*atrB* mutants have increased sensitivity to different classes of fungicides, mutagens and natural toxic compounds. Δ*atrD* mutants display increased sensitivity to cycloheximide, the cyclosporin derivative PSC 833, nigericin and valinomycin. These results indicate that AtrBp and AtrDp from *A. nidulans* are multidrug transporters with different substrate specifities (Chapters 3 and 4).

AtrBp has been further characterized by overexpression in *A. nidulans* and *S. cerevisiae* (Chapters 2, 4 and 6). Sensitivity to toxicants of a *PDR5*-deficient strain of *S. cerevisiae* was restored to wild-type levels, upon transformation with cDNA of *atrB* in a high copy number vector (Chapter 2). Mutants overexpressing *atrB* in *A. nidulans* also display decreased sensitivity to toxicants. These overexpression mutants display altered sensitivity to a wider range of compounds as compared to Δ*atrB* mutants (Chapter 4). These results indicate that the presence of additional drug-efflux pumps with affinity for the same compound prevent a change in phenotype of some deletion mutants. Redundancy of ABC transporters may explain, at least in part, the findings that Δ*atrA* and Δ*atrC* mutants show no hypersensitive phenotype for any of the compounds tested (Chapters 3 and 5). However, the observation that *atrA* transcript levels were not influenced by any of the compounds tested and that *atrA* expression in *S. cerevisiae* does not confer drug resistance, suggest that AtrAp is not a multidrug transporter.

ABC transporters which have overlapping substrate specificities may still have specific substrates. AtrBp has a distinctive specificity for the phenylpyrrole fungicide

fludioxonil since both Δ*atrB* and *atrB* overexpression mutants have altered sensitivity to this compound (Chapter 6). In addition, the increase in sensitivity to fludioxonil observed for Δ*atrB* mutants correlates with the relatively high accumulation levels of this compund. This was not observed for fenarimol. Therefore, the ABC transporter AtrBp can be regarded as a major efflux pump of phenylpyrrole fungicides in *A. nidulans*.

Unexpectedly, overexpression mutants of *atrB* displayed increased sensitivity to dithiocarbamate fungicides, chlorothalonil and the iron-activated antibiotic phleomycin (Chapter 4). This phenotype was most pronounced in the overexpression mutant with the highest levels of *atrB* expression. We hypothesize that this phenomenon could relate to involvement of AtrBp in iron metabolism.

Δ*atrD* mutants display a decrease in penicillin production, indirectly measured as antimicrobial activity against *Micrococcus luteus* (Chapter 3). These results suggest that AtrDp has a role in penicillin production.

In conclusion, data presented in this thesis demonstrated that some of the identified ABC transporters from *A. nidulans* function in protection against natural toxicants and xenobiotics. Deletion and overexpression mutants of specific *atr* genes display increased and decreased sensitivity to toxicants, respectively. A role for ABC transporters in production of fungal secondary metabolites has also been suggested. This may imply that strains overexpressing multidrug-transporter genes can show pleiotropic phenotypes with respect to production of secondary metabolites.

SAMENVATTING

Gelijktijdige resistentie van cellen tegen chemisch niet-verwante verbindingen wordt beschreven met de term multidrug resistentie (MDR). MDR gaat vaak gepaard met overproductie van multidrug-transport eiwitten van de ATP-bindings-cassette (ABC) superfamilie. ABC transporters vormen een grote en multifunctionele eiwitfamilie. Behalve multidrug transporters bevat de superfamilie ook eiwitten die betrokken zijn bij transmembraan transport van diverse stoffen zoals ionen, aminozuren, peptiden, suikers, vitaminen, steroïd hormonen, galzuren en fosfolipiden. Een overzicht van de grote verscheidenheid in cellulaire functies die deze eiwitten in levende cellen kunnen bezitten, wordt gegeven in Hoofdstuk 1.

Het doel van dit proefschrift was om moleculaire mechanismen verantwoordelijk voor drug resistentie in *Aspergillus nidulans* te identificeren en te karakteriseren. Hierbij werd de aandacht vooral gericht op drug-efflux eiwitten van de ABC superfamilie. Met behulp van diverse technieken hebben we zeven ABC-transporter genen (*atrA-G*) van *A. nidulans* geïdentificeerd. Heterologe screening van een genomische bibliotheek van *A. nidulans* met een DNA probe van *PDR5*, een goed gekarakteriseerde multidrug transporter van *Saccharomyces cerevisiae*, leidde tot de ontdekking van *atrA* en *atrB*, de eerste ABC-transporter genen die uit een filamenteuze schimmel zijn geïsoleerd (Hoofdstuk 2). De tweede benadering, PCR met gedegenereerde primers gebaseerd op consensus sequenties voor ABC-transporter genen uit de subfamilie van P-glycoproteïnen, resulteerde in de klonering van *atrC* en *atrD* (Hoofdstuk 3). De laatste benadering was gebaseerd op screening van een EST bibliotheek van *A. nidulans* op aanwezigheid van sequenties coderend voor eiwitten met homologie voor bekende multidrug transporters in schimmels, in het bijzonder ABC transporters betrokken bij efflux van azool fungiciden. Met deze benaderingswijze werden *atrE*, *atrF* en *atrG* geïdentificeerd (Hoofdstuk 5). De eiwitten die door alle zeven *atr* genen worden gecodeerd, bezitten een hoge mate van homologie met multidrug efflux eiwitten van andere organismen.

Om de rol van de geïdentificeerde *atr* genen van *A. nidulans* in multidrug transport te bestuderen, is de expressie van deze genen bestudeerd in het wild-type en MDR (*ima*) mutanten van *A. nidulans* na behandeling met toxische verbindingen. *imaA* en *imaB* zijn mutanten van *A. nidulans* die in het laboratorium zijn geselecteerd op resistentie tegen het azool fungicide imazalil en verwante verbindingen. In *imaB*

mutanten, is resistentie tegen het verwante fungicide fenarimol gebaseerd op een toename in energie-afhankelijke efflux activiteit, hetgeen resulteert in een afname van de accumulatie van de stof in het cytoplasma. Deze mutanten waren derhalve bruikbaar om resistentie tegen azolen te correleren met expressie niveaux van *atr* genen.

Expressiestudies in een wild-type isolaat toonde aan dat het basale niveau van expressie voor de meeste *atr* genen laag is en sterk verhoogd kan worden door behandeling met niet-verwante verbindingen (Hoofdstukken 2, 3, 5 en 6). Al 5 minuten na behandeling met toxische verbindingen (b.v. imazalil) kon reeds een toename in transcriptie niveau van *atr* genen worden waargenomen (Hoofdstuk 2). Sommige verbindingen induceren specifiek de transcriptie van één *atr* gen terwijl andere de transcriptie van meerdere *atr* genen beïnvloeden (Hoofdstuk 5). Resveratrol induceert bijvoorbeeld specifiek de transciptie van *atrB*, terwijl fenarimol de transcriptie verhoogt van meerdere genen (*atrB-G*). Expressie analyse van *ima* mutanten van *A. nidulans* toonde aan dat *atrD*, *atrE*, *atrF* en *atrG* een hoger niveau van basale expressie vertonen in *ima* mutanten dan in het wild-type (Hoofdstuk 5). Behandeling met fenarimol verhoogt ook het transcriptie niveau van deze *atr* genen in *imaB* mutanten.

Mutanten waarin *atrB* en *atrD* zijn uitgeschakeld vertonen een verhoogde gevoeligheid voor een aantal niet-verwante verbindingen. Δ*atrB* mutanten bezitten een toename in gevoeligheid voor verschillende groepen van fungiciden, mutagentia en natuurlijk toxische verbindingen. Δ*atrD* mutanten bezitten een toename in gevoeligheid voor cycloheximide, het cyclosporine derivaat PSC 833, nigericine en valinomycine. Deze resultaten duiden er op dat AtrBp en AtrDp van *A. nidulans* multidrug transporters zijn met een verschillende substraat specificiteit (Hoofdstukken 3 en 4).

AtrBp is verder gekarakteriseerd door overexpressie in *A. nidulans* en *S. cerevisiae* (Hoofdstukken 2, 4 en 6). Transformatie met cDNA van *atrB* in een multicopy vector naar een *PDR5*-disruptie mutant van *S. cerevisiae* resulteerde in transformanten met een wild-type gevoeligheid voor toxische verbindingen (Hoofdstuk 2). Dezelfde afname in gevoeligheid voor toxische verbindingen werd gevonden in AtrBp overexpressie mutanten van *A. nidulans*. Deze *atrB* overexpressie mutanten vertonen voor meer verbindingen een andere gevoeligheid dan de Δ*atrB* mutanten (Hoofdstuk 4). Deze resultaten wijzen er op dat de aanwezigheid van additionele drug-efflux pompen met affiniteit voor dezelfde verbinding verandering van het fenotype van sommige deletie mutanten kan voorkomen. De aanwezigheid van meerdere ABC

transporters kan, op zijn minst ten dele, de waarnemingen verklaren dat *ΔatrA* en *ΔatrC* mutanten niet overgevoelig zijn voor de geteste verbindingen (Hoofdstuk 3 en 5). De waarneming dat het *atrA* transcriptie niveau door geen van de geteste verbindingen wordt beïnvloed en dat *atrA* expressie in *S. cerevisae* geen resistentie tegen drugs bewerkstelligt, suggereert dat AtrAp geen multidrug transporter is.

ABC transporters met een overlappende substraat specificiteit kunnen toch nog specifiek zijn voor bepaalde substraten. AtrBp heeft een onderscheidende specificiteit voor het fenylpyrrol fungicide fludioxonil omdat zowel *ΔatrB* mutanten als *atrB* overexpressie mutanten een veranderde gevoeligheid voor deze verbinding bezitten (Hoofdstuk 6). Bovendien correleert de toename in gevoeligheid van *ΔatrB* mutanten voor fludioxonil met een relatief hoog accumulatieniveau van het fungicide in de schimmel. Dit werd niet waargenomen voor fenarimol. Daarom kan de ABC transporter AtrBp worden beschouwd als de belangrijkste efflux pomp van fenylpyrrol fungiciden in *A. nidulans*.

Het is opvallend dat overexpressie mutanten van *atrB* een toename in gevoeligheid vertonen voor dithiocarbamaat fungiciden, chloorthalonil en het antibioticum fleomycine dat door ijzer wordt geactiveerd. (Hoofdstuk 4). Het fenotype was het duidelijkst in overexpressie mutanten die het hoogste niveau van *atrB* expressie vertonen. We veronderstellen dat dit fenomeen te maken kan hebben met de rol van AtrBp in ijzer metabolisme.

ΔatrD mutanten vertonen een afname in penicilline productie, indirect gemeten als antimicrobiële activiteit tegen *Micrococcus luteus* (Hoofdstuk 3). Deze resultaten suggereren dat AtrDp een rol vervult bij penicilline productie.

Samenvattend tonen de gegevens die in dit proefschrift worden gepresenteerd aan, dat ABC transporters van *A. nidulans* kunnen functioneren bij de bescherming van cellen tegen natuurlijk toxische verbindingen en xenobiotica. Deletie en overexpressie mutanten van specifieke *atr* genen vertonen respectievelijk een toename en afname in gevoeligheid voor toxische verbindingen. De suggestie wordt ook gedaan dat ABC transporters een rol kunnen spelen bij de productie van secundaire metabolieten van schimmels. Dit zou kunnen betekenen dat stammen die multidrug-resistentie genen tot overexpressie brengen pleiotrope effecten vertonen met betrekking tot de productie van secundaire metabolieten.

Curriculum vitae

Alan Carvalho Andrade was born on June 9, 1968 in Lavras (Minas Gerais), Brazil. In 1990 he obtained his BSc degree in Agronomy, at the Universidade Federal de Lavras (UFLA). At the same University, he obtained his MSc in Agricultural Sciences, subject area Seed Physiology, in 1994. In the same year, the Fundação Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES-Brazil), awarded him a 4-year-grant to start his PhD study in the Netherlands. In April 1995, he started his PhD programme at the Laboratory of Phytopathology of Wageningen University, where the research described in this thesis was carried out. This research was also supported by a 1-year fellowship, granted by Eli Lilly and Company (Division of Infectious Diseases, Indianapolis-IN, USA). Since April 2000, he has a post-doc position at the Laboratory of Phytopathology, to work on the STW project WFA-5111, entitled "Development of ABC-transporter mutants of *Aspergillus nidulans* as innovative tools in drug discovery and secretion of secondary metabolites".

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