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

Alan C. Andrade,[1] Giovanni Del Sorbo,[2] Johannes G. M. Van Nistelrooy[1] and Maarten A. De Waard[1]

Author for correspondence: Maarten A. De Waard. Tel: +31 317 483412. Fax: +31 317 483412. e-mail: maarten.dewaard@medew.fyto.wau.nl

[1] Laboratory of Phytopathology, Wageningen University, PO Box 8025, 6700 EE Wageningen, The Netherlands

[2] Institute of Plant Pathology, University of Naples 'Federico II', 80055 Portici, Naples, Italy

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, strobirulin and some azole fungicides. Increased sensitivity to the natural toxic compounds camptothecin (alkaloid), the phytoalexin resveratrol (stilbene) and the mutagen 4-nitroquinoline 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 dithiocarbamate 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.

Keywords: multidrug resistance, efflux pumps, ABC transporters, AtrBp, *Aspergillus nidulans*

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 for 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 & De Waard, 1993). This has been

Abbreviations: ABC, ATP-binding cassette; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; EC_{50}, effective concentration required for 50% growth inhibition; MDR, multidrug resistance; 4NQO, 4-nitroquinoline oxide; UTR, untranslated region.

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 the development of MDR 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 ATP-binding cassette (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 & Van Nistelrooy, 1979, 1980). This mechanism also operates in plant pathogens such as *Penicillium italicum*, *Botrytis cinerea*, *Nectria haematococca*, and probably *Mycosphaerella graminicola* (De Waard *et al*., 1995; 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*., 1998; Urban *et al*., 1999). A high degree of homology also exists with ABC proteins classified in subcluster I.1 from *Saccharomyces cerevisiae* (Decottignies & 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 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.

METHODS

Strains, plasmids and media. The *A. nidulans* strains and plasmids 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). *Escherichia 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 d at 37 °C, were used as inoculum source for liquid cultures at a density of 10^7 conidia ml^{-1}. Germlings harvested after 14 h incubation at 37 °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) was used to generate radioactively labelled oligonucleotide probes with [α-^{32}P]dATP. Southern, Northern and dot blot hybridizations were performed using Hybond N^+ (DNA) and Hybond N (RNA) nylon membranes (Amersham), according to the manufacturer's instructions. Overnight hybridized blots were washed at 65 °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). Sequences were analysed using the DNAstar package (DNASTAR).

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 *Aspergillus 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

Table 1. *A. nidulans* strains and plasmids 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	
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	
Plasmid	**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	γ-Actin of *A. nidulans* cloned in pUC19	Fidel *et al.* (1988)
pGspD	Subclone containing *atrD* gene-specific probe	Andrade *et al.* (2000)
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.

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 (MM) 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 °C and 300 r.p.m. in an orbital incubator for 16 h. The germlings were harvested through Mira-Cloth, washed twice with sterile water and twice with STC buffer (1·0 M sorbitol, 10 mM Tris/HCl pH 7·5, 50 mM $CaCl_2$) and squeezed between paper towels to remove excess of liquid. Protoplasts were released by incubation of 1 g mycelium at 30 °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 h. 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 °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 & Van Nistelrooy, 1979). Mycelial agar plugs of an overnight-grown confluent plate of each strain were placed upside down on MM plates amended with fungicides at different concentration of the compounds. Radial growth was assessed after 3 d incubation, at 37 °C. Carbendazim and sulfomethurom methyl were kindly provided by DuPont De Nemours, cilofungin by Eli

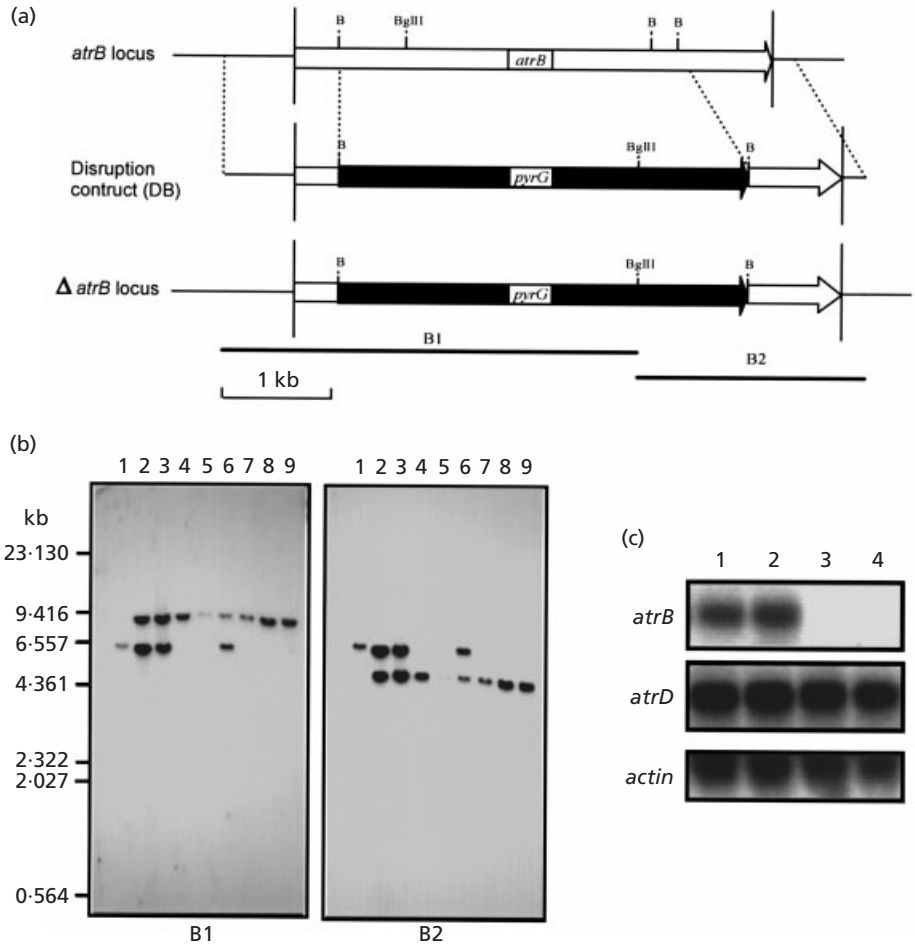

Fig. 1. Generation of deletion mutants of the *A. nidulans atrB* gene. (a) Schematic representation of the wild-type *atrB* locus, disruption construct and knock-out locus of *atrB* . Horizontal lines labelled B1 and B2 indicate the restriction fragments used as probes in Southern analysis. (b) Southern 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 *Bgl*II 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 shows the same blot hybridized with the gene-specific probe GspD of *atrD* and the bottom panel with actin as loading controls.

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. 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}), sodium *o*-phenylphenate (15 μg ml^{-1}), nystatin (10 μg ml^{-1}), 4-nitroquinoline 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 & 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 & Van Nistelrooy, 1980).

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. 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.

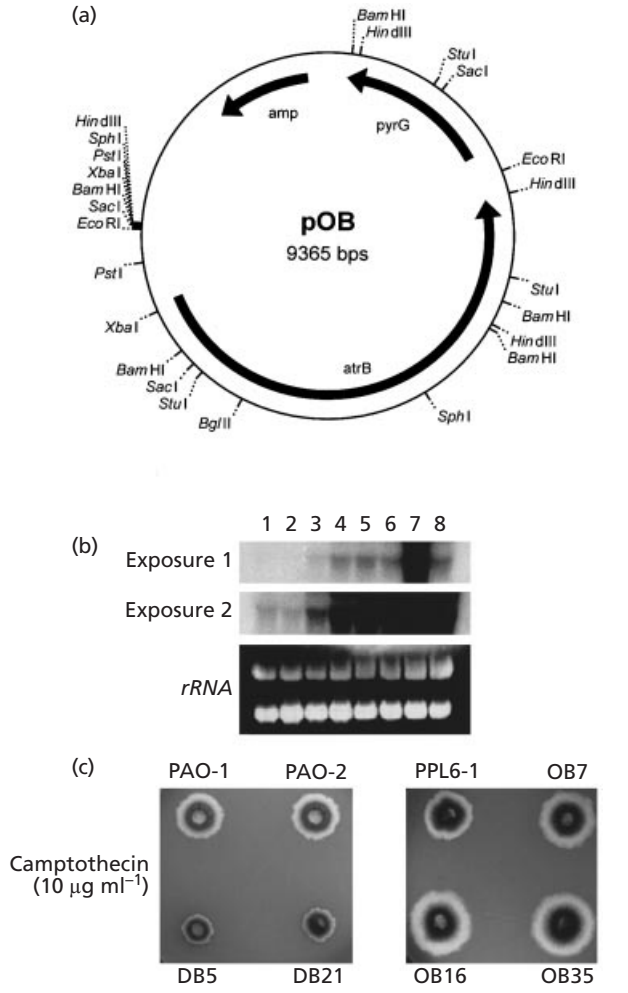

Fig. 2. Generation of overexpression mutants of the *A. nidulans atrB* gene. (a) Schematic representation of the pOB construct used for transformation. Restriction sites are indicated. (b) Northern blot analysis of RNA isolated from untreated germlings of *A. nidulans* after different exposure times 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).

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′ UTR. This construct was cloned in the pPL6 vector (Oakley *et al*., 1987), which contains the *pyrG* gene from *A. nidulans*. A schematic representation of the transformation construct coded by 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 Fig. 2c).

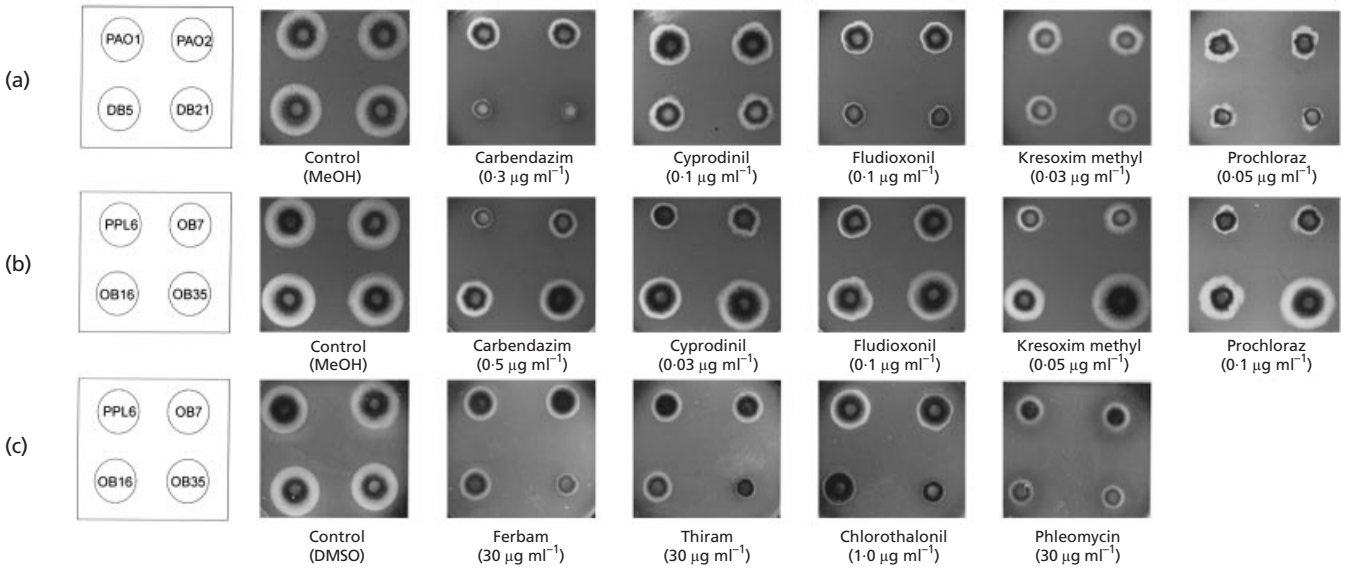

Fig. 3. Altered sensitivity of deletion and overexpression mutants of *atrB* from *A. nidulans* to fungicides and natural toxins. (a) Control strains PAO-1 and PAO-2 and *atrB* deletion mutants DB-5 and DB-21 showing increased sensitivity to toxic compounds. (b) Control strain PPL6-1 and overexpression 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.

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 Methods) 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 2 and 3). 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).

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 2 and 3). Furthermore, the degree of decreased sensitivity to these compounds was positively correlated with the level of *atrB* expression (Fig. 3b, Tables 2 and 3). 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 2 and 3).

AtrB 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 & 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 the control strain PPL6-1 (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 (orthovanadate) 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 & Van

Table 2. Sensitivity of deletion (DB5 and DB21) and overexpression mutants (OB7, OB16 and OB35) of *atrB* in *A. nidulans* to fungicides and antimycotics in radial growth tests

Fungicide or antimycotic	EC_{50} Pao* (mg l^{-1})	Q_{50}† Δ*atrB*	EC_{50} Ppl6* (mg l^{-1})	Q_{50}†		
				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						
Sodium *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 Methods) are statistically different according to Tukey's test ($P < 0·05$).

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 (Fig. 4d).

Table 3. Sensitivity of deletion (DB5 and DB21) and overexpression mutants (OB7, OB16 and OB35) of *atrB* in *A. nidulans* to natural toxic products and miscellaneous compounds in radial growth tests

Compound	EC_{50} Pao* (mg l^{-1})	Q_{50}† Δ*atrB*	EC_{50} Ppl6* (mg l^{-1})	Q_{50}†		
				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 the solubility level of the compound and could not be accurately determined.

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.

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 (phenylpyridianine) and azoxystrobin, kresoxim-methyl and trifloxystrobin (strobirulins). Increased sensitivity was also observed for other compounds such as 4-nitroquinoline 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, 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 *Saccharomyces 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

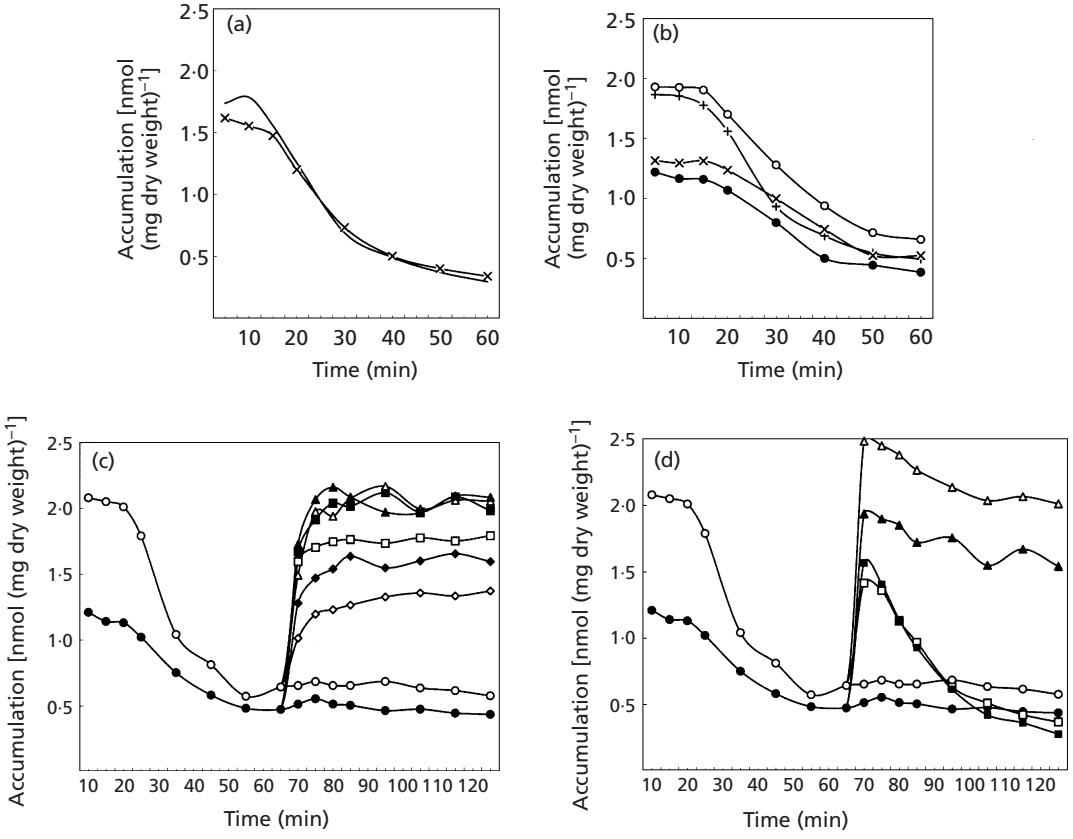

Fig. 4. Accumulation of [^{14}C]fenarimol by germlings of *A. nidulans*. (a) Accumulation of [^{14}C]fenarimol (30 μM) by control strains PAO (bold line) and Δ*atrB* mutants (×). (b) Accumulation of [^{14}C]fenarimol by control strain PPL6-1 (○) and the *atrB* overexpression mutants OB7 (+), OB16 (×) and OB35 (●). (c) Effect of CCCP (□, ■), oligomycin (△, ▲) and sodium orthovanadate (◇, ◆) 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 orthovanadate (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%; ○, ●).

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 Δ*atrB* mutants and control strains is similar. In constrast, 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 Δ*atrB* 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 (orthovanadate), 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 subcluster I.1 (Decottignies & 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 subcluster. 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*., 1998). 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 suggests 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 Δ*atrB* 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 & Sharom, 1998; Sharom, 1997). Hence, differences in membrane composition of yeast as compared to *A. nidulans* could explain these results.

Most interestingly, the overexpression mutants of *atrB* displayed increased sensitivity to dithiocarbamate 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.

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