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A. C. Andrade · J. G. M. Van Nistelrooy
R. B. Peery · P. L. Skatrud · M. A. De Waard

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

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Abstract 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 *A. fumigatus*. Northern 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 *pyrG* from *A. 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.

Key words *Aspergillus nidulans* · ABC transporters · Multidrug resistance · Antibiotic secretion

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A. C. Andrade · J. G. M. Van Nistelrooy
M. A. De Waard (✉)
Laboratory of Phytopathology,
Wageningen University, P.O. Box 8025,
6700 EE Wageningen, The Netherlands
E-mail: maarten.dewaard@medew.fyto.wau.nl
Tel.: +31-317-483123; Fax: +31-317-483412

R. B. Peery · P. L. Skatrud
Eli Lilly and Company, Infectious Diseases Research,
Lilly Corp. Center, Indianapolis, IN 46285, USA

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 the 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 well known for their role in multidrug resistance (MDR) in human tumor 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-tumor 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 Facilitator (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* (Nishi et al. 1992; Nagao et al. 1995; 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 is 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 characterization 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 to be 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 from *A. fumigatus* and *A. 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 (Mahe et al. 1996; Van Helvoort et al. 1996; Decottignies et al. 1998; Kamp and Haest 1998). It has been suggested that fungal ABC transporters might 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 across biological membranes (Brakhage 1998; Penalva et al. 1998; Brown et al. 1999).

In this paper we report on the isolation and functional characterization 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.

Materials and methods

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 the host for plasmid propagation.

Table 1 *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 acid manipulations and molecular biological techniques

Freshly harvested conidia obtained from confluent plate cultures of *A. nidulans*, grown for 4–5 days at 37 °C, were used as a source of inoculum for liquid cultures, at a density of 10^7/ml. Germlings harvested after 14 h of incubation at 37 °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 Qiagen kit (Qiagen, Chatsworth, Calif.). cDNA synthesis was performed using the Marathon cDNA amplification kit with the Advantage cDNA polymerase mix (Clontech, Palo Alto, Calif.). The Random Primer DNA Labelling System (GIBCO-BRL, 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 Hybond N$^+$ (DNA) and Hybond N (RNA) nylon membranes (Amersham, Little Chalfont, Bucks., UK), according to manufacturer's instructions. Blots were hybridized overnight and washed at 65 °C with 0.1% SSC/0.1% SDS. The vectors pGEM-3Zf(+) and pGEM-T (Promega, Madison, Wis.) 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 was performed using a Perkin-Elmer DNA Thermal Cycler 480 and Amplitaq DNA polymerase (Perkin Elmer, Branchburg, N.J.), unless otherwise indicated. Sequences were analyzed using the DNAstar package (DNASTAR).

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 sequences similar to those of 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). The primer aspmdr-1 (5′-GCYCTCGTYGGICCCTCIGG-3′) or aspmdr-3 (5′-GCYCTCGTYGGICCCAGYGG-3′), corresponding to the amino acid sequence ALVGPSG, was used in combination with aspmdr-2 (5′-GATRCGYTGCTTYTGICCICC-3′), the complement of the sequence encoding GGQKQRI. PCRs involved 30 cycles of melting at 94 °C for 30 s, annealing at 60 °C for 30 s, and extension at 72 °C for 20 s. Reaction products were amplified again by transferring 2 µl of the original reaction into a fresh PCR mix and allowing the reaction to proceed under the same conditions.

Genomic library

A wild-type genomic library constructed in λEMBL3 was used (Del Sorbo et al. 1997). Positive lambda clones were screened and subjected to at least three rounds of plaque purification.

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 resulting 3.9-kb DNA fragment was used to obtain a 3.8-kb *Bam*HI insert from pAO4-2 by restriction 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 into the *Bam*HI site of pC7O4, giving rise to a 9.0-kb construct, termed 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 by digestion of pAO4-2 with *Bam*HI and *Bgl*II into the *Bam*HI site of subclone D30, which contained a 0.4-kb *Eco*RI-*Bam*HI insert. This construct was named pD30O4. In the second step, a 2.0-kb *Bgl*II fragment obtained from subclone D26 by digestion with *Bgl*II was cloned in the *Bam*HI site of pD30O4, resulting in a 8.4-kb construct designated pAOD. The final transformation construct of 5.2 kb was obtained by 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 casamino acids, 0.5 g/l yeast extract and the required auxotrophic nutrients was inoculated with 10^6 conidia/ml and incubated overnight at 37 °C and 300 rpm in a 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-Cl pH 7.5, 50 mM $CaCl_2$), and excess liquid was removed by blotting between paper towels. Protoplasts were released by incubation of 1 g of mycelium at 30 °C and 100 rpm, resuspended in 20 ml of filter-sterilized iso-osmotic S0.8MC medium containing lytic enzymes (5 mg/ml Novozym 234, 0.8 M KCl, 50 mM $CaCl_2$, 20 mM MES pH 5.8) for about 2 h. The protoplast solution was filtered through glass wool, diluted (1:1) with STC buffer and incubated on ice for 10 min. Then, protoplasts were collected by centrifugation (10 min, 0 °C, 3000 rpm) and washed twice with STC buffer. Transformation was performed as described by Van Heemst et al. (1997) using the purified transformation constructs DC and DD (3.5 μg) dissolved in sterile water (15 μl).

Toxicity assays

Sensitivity of *A. nidulans* strains to toxic agents was determined in a radial growth test on MM plates (De Waard and Van Nistelrooy 1979). Benomyl and sulfomethurom methyl were kindly provided by DuPont De Nemours (Wilmington, Del.), bitertanol by Bayer AG (Leverkusen, Germany), cilofungin by Eli Lilly and Co. (Indianapolis, Ind.), the cyclosporin derivative PSC 833 by Novartis (Basel, Switzerland), fenarimol by Dow Elanco (Greenfield, Ind.) 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 (Zwijndrecht, The Netherlands). The following test chemicals were dissolved in agar at the indicated test concentations: actinomycin D (100 μg/ml), benomyl (1 μg/ml), bergenin (100 μg/ml), bitertanol (10 μg/ml), cycloheximide (25 μg/ml), cyclosporin derivative PSC 833 (25 μg/ml), chlorpromazine (25 μg/ml), chloramphenicol (100 μg/ml), eugenol (2.5 μl/ml), fenarimol (3 μg/ml), genistein (100 μg/ml), gramicidin D (10 μg/ml), imazalil (0.03 μg/ml), kresoxim methyl (0.3 μg/ml), nigericin (3 μg/ml), nystatin (10 μg/ml), 4-nitroquinoline oxide (1 μg/ml), oligomycin (0.25 μg/ml), pisatin (20 μg/ml), quinidine (200 μg/ml), resveratrol (200 μg/ml), rhodamine 6G (5 μg/ml), triflupromazine (10 μg/ml), tomatine (10 μg/ml), valinomycin (3 μg/ml). These compounds were added from concentrated solutions in methanol. Amphotericin B (25 μg/ml), brefeldin A (5 μg/ml), camptothecin (25 μg/ml), cilofungin (0.1 μg/ml), psoralen (200 μg/ml), quercetin (200 μg/ml), and sulfomethuron methyl (100 μg/ml) were added from concentrated solutions in DMSO. Acriflavin (1 μg/ml), ethidium bromide (1 μg/ml) and neomycin sulfate (600 μg/ml) 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* and 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 °C. The strain of *Micrococcus luteus* (DSM-348) was purchased from DSMZ (Braunschweig, Germany). Overnight bacterial cultures were grown on Lab-Lemco Broth (Oxoid) at 30 °C and 200 rpm. In bioassays, portions (50 ml) of freshly prepared sterile Lab-Lemco Agar (Oxoid) were cooled to 45 °C, mixed with overnight bacterial culture (1 ml) and tranferred to 145 mm plates. Then, agar plugs from the center of 14-day-old colonies of the *A. nidulans* strains to be tested were placed equidistantly on top of the bacterial plates and incubated at 30 °C. Inhibition zones were measured after 24 h of incubation.

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 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 (ABC transporter 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

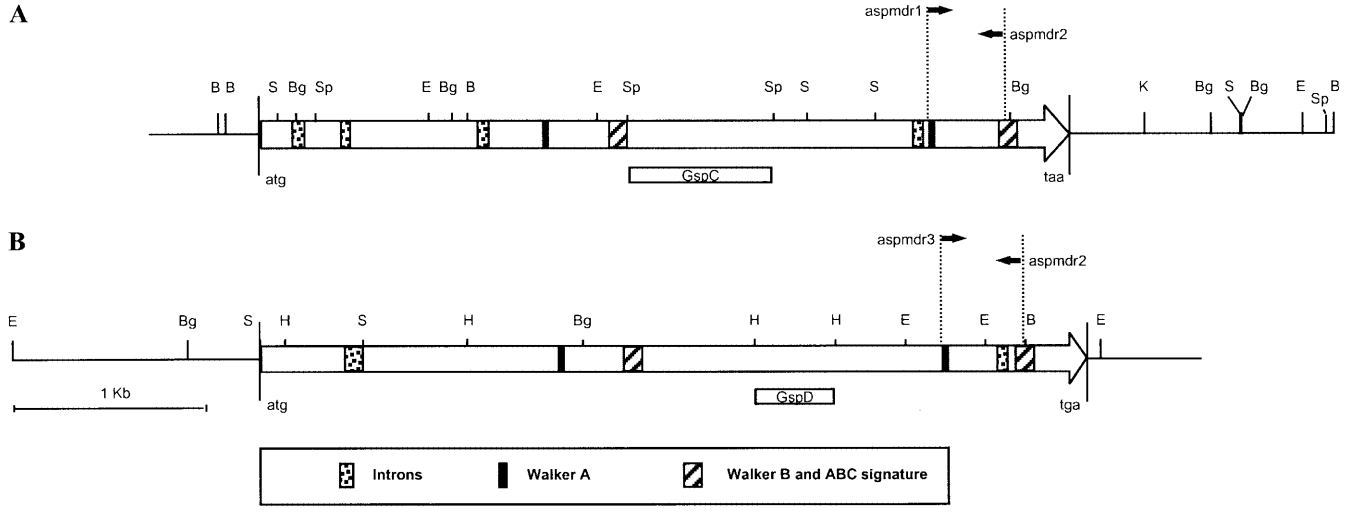

Fig. 1A, B Physical map of the genomic regions encoding *atrC* (**A**) and *atrD* (**B**). The *open arrows* represents the coding regions of the *atr* genes interrupted by introns. The conserved motifs characteristic of ABC transporter proteins are shown. The PCR fragments amplified using degenerate 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 indicated as follows: B, *Bam*HI; Bg, *Bgl*II; E, *Eco*RI; H, *Hind*III; K, *Kpn*I; S, *Sal*I and Sp, *Sph*I

of the phage library with this fragment resulted in the isolation of 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 2). 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 2), 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 in Fig. 2 represent amino acids that interact with ATP (Hung et al. 1998) and are highly conserved in other ABC transporters.

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 B, respectively.

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*

Table 2 Pairwise comparison of deduced amino acid sequences of *atrC* and *atrD* from *Aspergillus nidulans* with other ABC transporters of the P-glycoprotein subfamily

Protein	Organism	GenBank No.	AtrCp[a]			AtrDp[a]		
			BLAST score[a]	Identity (%)	Similarity (%)	BLAST score[a]	Identity (%)	Similarity (%)
AtrCp	*Aspergillus 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	*Schizosaccharomyces pombe*	P36619	0	36	51	0	45	60
CneMdr1p	*Cryptococcus neoformans*	U62930	0	34	51	0	42	57
ChMdr1p	*Cricetulus griseus*	P21448	0	33	51	0	38	55
HsMDR1	*Homo sapiens*	P08183	0	32	50	0	39	55
HvMdr2p	*Hordeum vulgare*	Y10099	e^{-158}	31	48	0	34	51
Ste6p	*Saccharomyces cerevisiae*	P12866	e^{-84}	24	43	e^{-113}	24	43

[a] Results were obtained using the BLAST program for sequence alignment (Altschul et al. 1997). Where indicated the BLAST score gives the probability that the match observed is due to chance

Fig. 2 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 detected by Decottignies and Goffeau (1997) are *highlighted* against a gray background. Residues in *bold* interact with ATP (Hung et al. 1998)

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N-terminal                    A                   C                   B                   D
An AtrCp (AF071410)  LVGQSGSGKSTIV QQEP LSGGQKQRVAIARSVVSQPKVLLLDEATSALD TTIVIAHKLAT
An AtrC2p*(AF082072) IVGSSGSGKSTIL QQEP LSGGQKQRIAIARSIIRNPPILLLDEATSALD TTIIVAHRLST
An AtrDp (AF071411)  LVGPSGSGKSTVV SQEP LSGGQKQRIAIARAVVSDPKILLLDEATSALD TTIVIAHRLST
Afu Mdr1p(U62933)    LVGPSGSGKSTVV SQEP LSGGQKQRIAIARAIVSDPKILLLDEATSALD TTIVIAHRLST
Afl Mdr1p(U62931)    FVGPSGSGKSTII SQEP LSGGQKQRIAIARAIIKDPKILLLDEATSALD TTIVIAHRLST
Sp Pmd1p  (P36619)   LVGASGSGKSTII QQEP MSGGQKQRIAIARAVISDPKILLLDEATSALD TTIVIAHRLST
Sc Ste6p  (P12866)   IVGKSGSGKSTLS EQRC LSGGQQQRVAIARAFIRDTPILFLDEAVSALD TTIILTHELSQ
Hs MDR1   (P08183)   LVGNSGCGKSTTV SQEP LSGGQKQRIAIARALVRNPKILLLDEATSALD TTIVIAHRLST
Ll Lmrap  (U63741)   FAGPSGGGKSTIF SQDS ISGGQRQRLAIARAFLRNPKILMLDEATASLD TTLVIAHRLST
St HisP   (P02915)   IIGSSGSGKSTFL NQLR LSGGQQQRVSIARALAMEPDVLLFDEPTSALD TMVVVTHEMGF
                     : * ** **** .*  :****:**::***:. :. :*::**.::** * ::::*.::
C-terminal
An AtrCp             FVGSSGCGKSTMI QQEP LSGGQRQRIAIARALIRDPKILLLDEATSALD LTVAVAHRLST
An AtrC2p*           LVGASGCGKTTVI TQNP LSGGQRQRIAIARALIRDPELLLFDEATSALD TTISVAHRLTT
An AtrDp             LVGPSGCGKSTTI SQEP LSGGQKQRVAIARALLRDPKILLLDEATSALD TTIAVAHRLST
Afu Mdr1p            LVGPSGCGKSTTI SQEP LSGGQKQRVAIARALLRDPKVLLLDEATSALD TTIAVAHRLST
Afl Mdr1p            LVGASGSGKSTTI SQEP LSGGQKQRIAIARALIRNPKILLLDEATSALD TTIAVAHRLST
Sp Pmd1p             FVGSSGCGKSTTI SQEP LSGGQKQRIAIARALIRNPKILLLDEATSALD TTVAIAHRLSS
Sc Ste6p             IIGESGTGKSTLV EQKP LSGGQAQRLCIARALLRKSKILILDECTSALD LTMVITHSEQM
Hs MDR1              LVGSSGCGKSTVV SQEP LSGGQKQRIAIARALVRQPHILLLDEATSALD TCIVIAHRLST
Ll Lmrap             FAGPSGGGKSTIF SQDS ISGGQRQRLAIARAFLRNPKILMLDEATASLD TTLVIAHRLST
St HisP              IIGSSGSGKSTFL NQLR LSGGQQQRVSIARALAMEPDVLLFDEPTSALD TMVVVTHEMGF
                     : * ** **:* .  *   :**** **:.****:  ...:*::** *::**   : ::*
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* 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*.

(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).

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

In order functionally to 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 gene (*pyrG*) of *A. oryzae*, using a uridine-auxotrophic mutant (WG488) of *A. nidulans* as the recipient strain for transformation. Selection of transformants was based on screening for uridine prototrophy. The use of the heterologous selectable marker from *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 from transformants were hybridized with the gene-specific probes GspC and GspD for *atrC* and *atrD*, respectively. Southern analysis of eight 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 for *atrC* (probe C1) and *atrD* (probe D1) was performed and confirmed the replacement of the wild-type locus (Fig. 4C and D). Northern 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 confirmed that *atrC* and *atrD* had been functionally deleted.

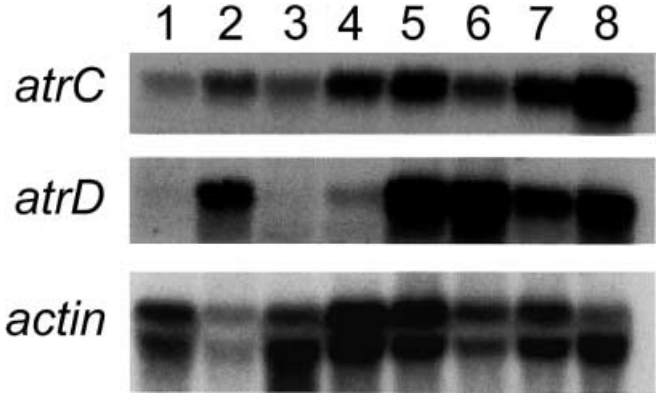

Fig. 3 Northern analysis of *atrC* (*top*) and *atrD* (*middle*) using total RNA from germlings of *Aspergillus nidulans* treated with toxicants for 60 min. Lane 1, control (0.1% DMSO); 2, reserpine (100 μg/ml); 3, sulfomethuron methyl (30 μg/ml); 4, control (0.1% ethanol); 5, imazalil (10 μg/ml); 6,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

Two independent monospore isolates of Δ*atrC* (DC-2 and DC-7) and Δ*atrD* (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. The activity of 35 compounds (see Materials and methods)

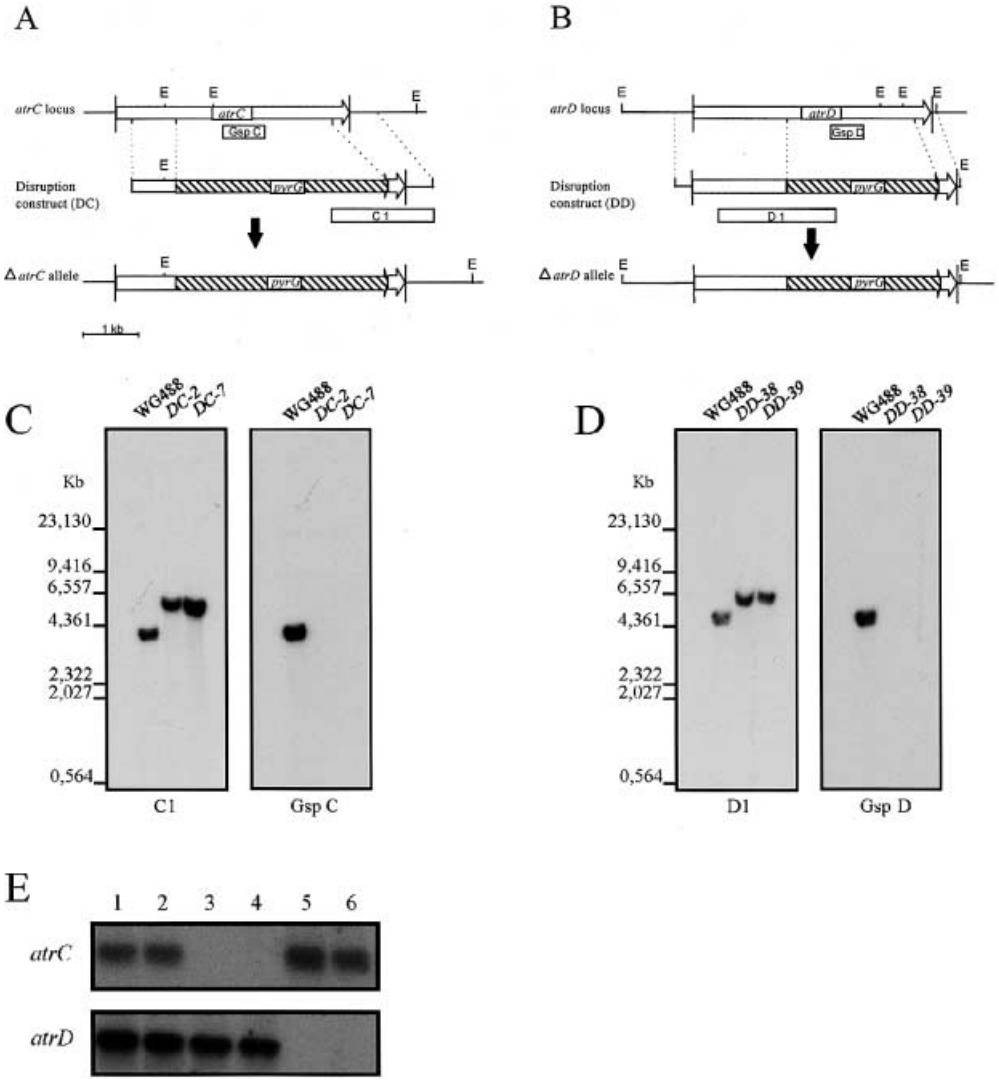

Fig. 4A–E Replacement of the *A. nidulans atrC* and *atrD* genes, and transcriptional analysis of the resulting mutants. **A, B** Schematic representation of the disruption construct, and wild-type and disrupted locus of *atrC* (**A**) and *atrD* (**B**). The *open boxes* labelled GspC, GspD, C1 and D1 indicate the restriction fragments used as probes in Southern and Northern analyses. **C** 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*. 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 bearing *Eco*RI-restricted genomic DNA of strains WG488, DD-38 and DD-39 was hybridized with the probes D1 and GspD. **E** Northern analysis of germlings of *A. nidulans* treated with cycloheximide (20 μg/ml) 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 for *atrC* and the *lower panel* shows the same blot hybridized (after stripping the first probe) with the gene-specific probe GspD for *atrD*

was tested. None of them differentially inhibited growth of the Δ*atrC* monospore isolates and control isolates PAO-1 and PAO-2. In contrast, Δ*atrD* mutants displayed increased sensitivity to cycloheximide (25 μg/ml), the cyclosporin derivative PSC 833 (25 μg/ml), nigericin (3 μg/ml), and valinomycin (3 μg/ml) 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, which 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 ability of identified substrates and transcriptional inducers of *atrD* to inhibit the efflux of [^{14}C]fenarimol. Pronounced inhibitory effects were observed with the cyclosporin derivative PSC 833, nigericin,

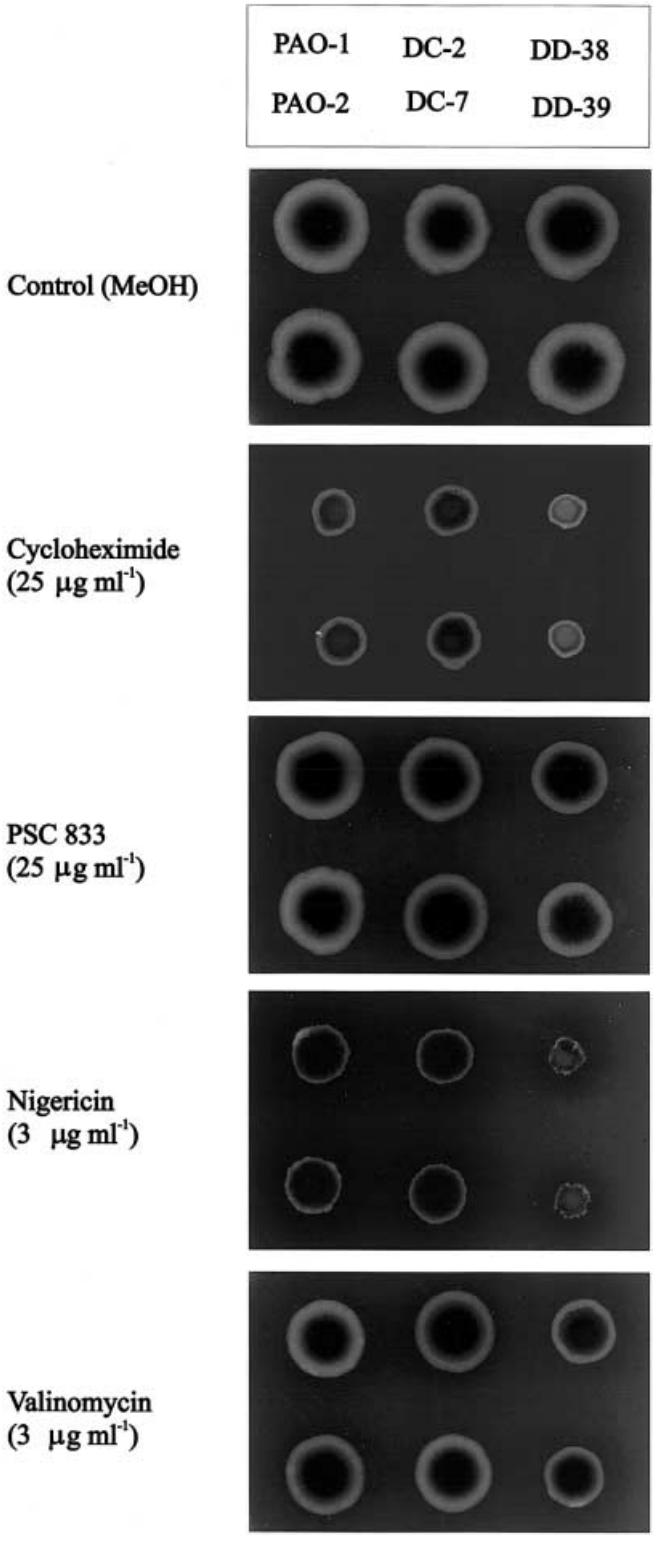

Fig. 5 Toxicity assays. Sensitivity of *A. nidulans* strains PAO-1 and PAO-2 (controls), DC-2 and DC-7 (Δ*atrC*) and DD-38 and DD-39 (Δ*atrD*) to four structurally unrelated compounds. Mycelial agar plugs of a confluent plate (incubated overnight) of each strain were placed upside down on a minimal medium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

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 drug. However, when applied 60 min prior to addition of the fungicide, inhibition of efflux activity was observed (data not shown). The parental strain PAO-2 and the *atrC* and *atrD* deletion mutants (DC-7 and DD-39, respectively) display a similar transient accumulation of [^{14}C]fenarimol (Fig. 6B).

Δ*atrD* 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 *M. luteus* as a test organism. Antibiotic activity of Δ*atrD* strains proved to be significantly lower (33% reduction) than that of control and Δ*atrC* strains (Fig. 7A and C). 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.

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 positions of introns in *atrD* and AfuMdr1p 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, Δ*atrD* 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 for the substrate specificity of ABC proteins is largely unknown. ABC transporters involved in MDR from various organisms can share a similar set of substrates but 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 differences in topology and low sequence homology (Kolaczkowski et al. 1996). Murine Mdr3p, PfMdr1p and human MRP are

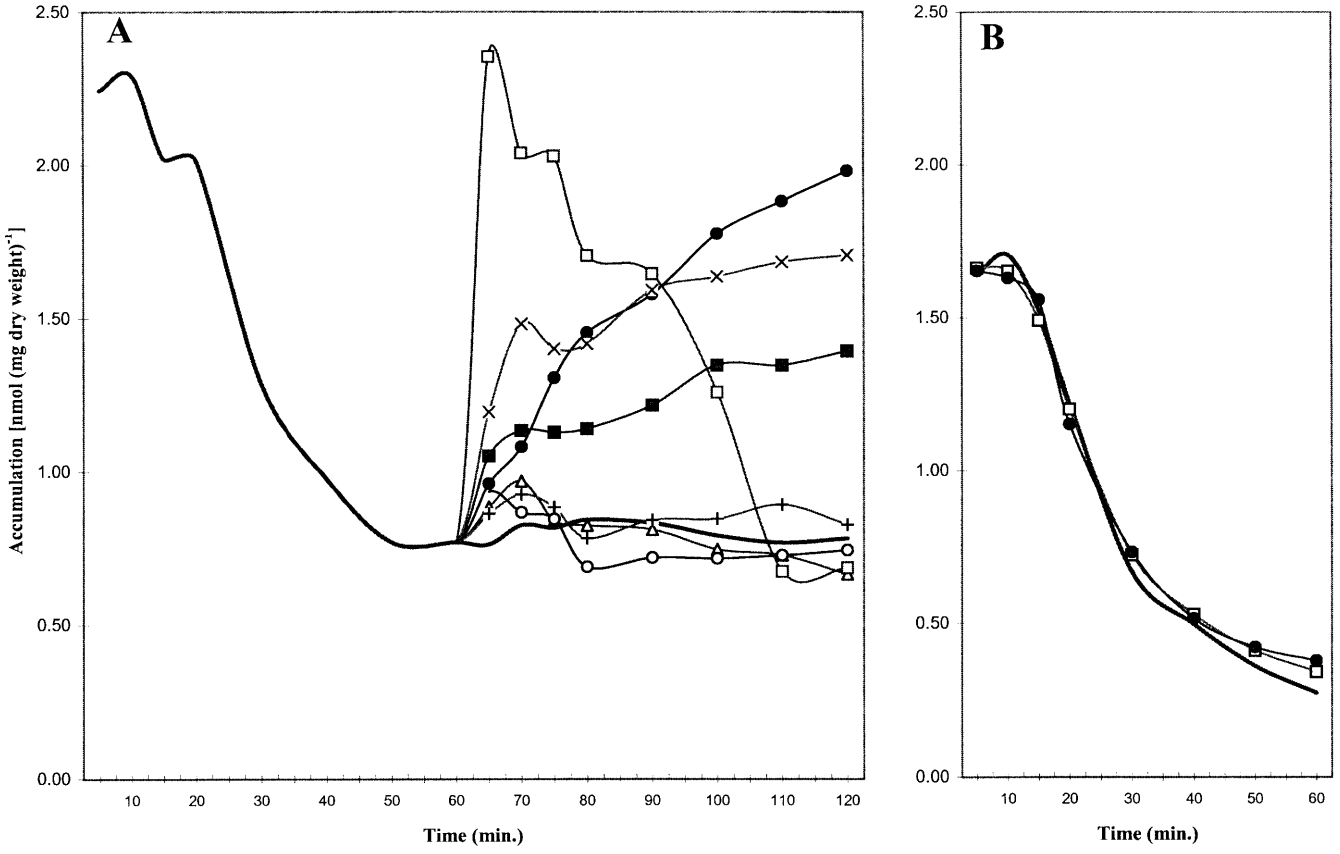

Fig. 6A, B Accumulation of [^{14}C]fenarimol by germlings of *A. nidulans*. **A** Effect of cycloheximide (*open triangles*), PSC 833 (*filled squares*), nigericin (*filled circles*), valinomycin (×), quercetin (*open circles*), and reserpine (*open squares*) on [^{14}C]fenarimol accumulation by germlings of strain PAO-2. Chemicals (at 100 μg/ml) 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 (*open squares*) and DD-39 (*filled circles*). [^{14}C]fenarimol was added to germlings at **t** = 0

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 the substrate specificity and ATPase activity of these proteins (Doige et al. 1993; Sharom 1997; Romsicki and Sharom 1998). 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 the 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 for AtrCp. However, the normal-growth phenotype observed for Δ*atrC* strains suggests that the role of *atrC* is not essential or can perhaps be provided by other ABC transporters. Increased levels of both *atrC* and *atrD* transcripts 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 these 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 Δ*atrC* mutants show no hypersensitivity phenotype for any of the set of compounds tested. This has also been

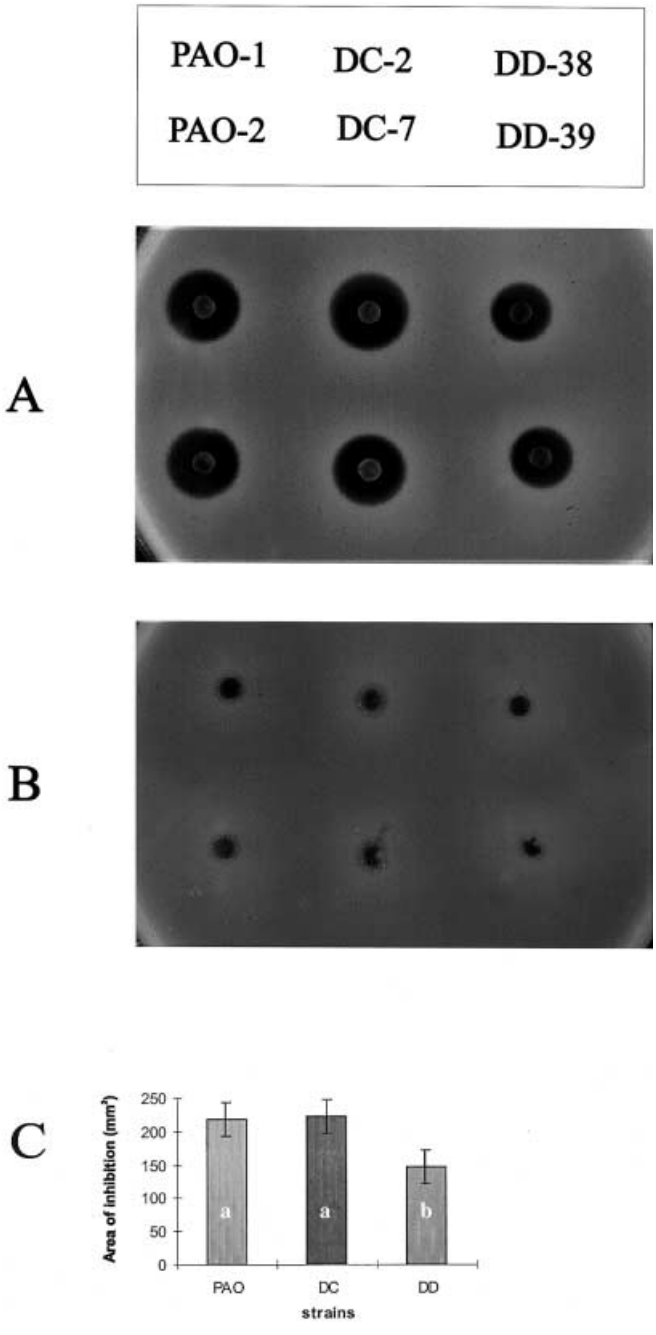

Fig. 7A–C Secreted antibiotic activity. Agar plugs taken from the center of 14-day-old colonies of *A. nidulans*, grown on complete medium (CM) plates at 25 °C, were placed on agar plates seeded with *Micrococcus luteus* and incubated overnight at 37 °C. **A** Inhibition zones indicating antibacterial 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** supplemented with penicillinase (10 units). **C** The *bars* represent the means of the area of inhibition (mm^2) obtained from six 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

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 Δ*atrD* 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 substrates for ABC transporters in other organisms (Kuchler et al. 1989; Nishi et al. 1992; Kolaczkowski et al. 1998; Seelig 1998; Ambudkar et al. 1999). Increased sensitivity to actinomycin D, as observed in a *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* (B. A. Roe, S. Kupfer, S. Clifton, R. Prade and J. Dunlap "*Aspergillus nidulans* and *Neurospora crassa* cDNA sequencing project: fifth data release", April 18 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. by 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 are similar. The cyclosporin derivative PSC 833, nigericin and valinomycin also induce accumulation of [^{14}C]fenarimol, but, in contrast to reserpine, their effect is not transient but proportional to the 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 by Δ*atrD* mutants suggests a role for 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 Δ*atrD* 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 involve 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, similar to the function proposed for the Ecs ABC transport proteins of *B. 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 for mammalian ABC transporters (Loe et al. 1997). The presence of the consensus binding motif 5′-TCG(N_5)CGA-3′ for AflR, a transcription factor involved in the 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 that overexpress 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 they enhance the secretion of detrimental compounds such as virulence factors or mycotoxins. For these reasons, ABC transporters in *Aspergilli* need further investigation.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res 25: 3389–3402

Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM (1999) Biochemical, cellular, and pharmacological aspects of the multidrug transporter. Annu Rev Pharmacol Toxicol 39: 361–398

Andrade AC, Zwiers L-H, De Waard MA (1999) ABC transporters and their impact on pathogenesis and drug sensitivity. In: Brooks GT, Roberts TR (eds) Pesticide chemistry and bioscience, the food-environment challenge. Royal Society of Chemistry, Cambridge, pp 221–235

Angermayr K, Parson W, Stoffler G, Haas H (1999) Expression of *atrC* – encoding a novel member of the ATP binding cassette transporter family in *Aspergillus nidulans* – is sensitive to cycloheximide. Biochim Biophys Acta 1453: 304–310

Atadja P, Watanabe T, Xu H, Cohen D (1998) PSC-833, a frontier in modulation of P-glycoprotein mediated multidrug resistance. Cancer Metastasis Rev 17: 163–168

Bairoch A (1992) PROSITE: a dictionary of sites and patterns in proteins. Nucleic Acids Res 20: 2013–2018

Balzi E, Wang M, Leterme S, Van Dyck L, Goffeau A (1994) PDR5, a novel yeast multidrug resistance-conferring transporter controlled by the transcription regulator PDR1. J Biol Chem 269: 2206–2214

Bissinger PH, Kuchler K (1994) Molecular cloning and expression of the *Saccharomyces cerevisiae* STS1 gene product. A yeast ABC transporter conferring mycotoxin resistance. J Biol Chem 269: 4180–4186

Brakhage AA (1998) Molecular regulation of beta-lactam biosynthesis in filamentous fungi. Microbiol Mol Bio Rev 62: 547–585

Brown MP, Brown Jenco CS, Payne GA (1999) Genetic and molecular analysis of aflatoxin biosynthesis. Fungal Genet Biol 26: 81–98

Clutterbuck AJ (1993) *Aspergillus nidulans* genetic loci. In: O'Brien SJ (ed) Genetic maps. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, pp 3.71–3.84

Cole SP, Bhardwaj G, Gerlach JH, Mackie JE, Grant CE, Almquist KC, Stewart AJ, Kurz EU, Duncan AM, Deeley RG (1992) Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line. Science 258: 1650–1654

De Ruiter Jacobs YM, Broekhuijsen M, Unkles SE, Campbell EI, Kinghorn JR, Contreras R, Pouwels PH, van den Hondel CA (1989) A gene transfer system based on the homologous *pyrG* gene and efficient expression of bacterial genes in *Aspergillus oryzae*. Curr Genet 16: 159–163

De Waard MA (1997) Significance of ABC transporters in fungicide sensitivity and resistance. Pestic Sci 51: 271–275

De Waard M, Van Nistelrooy J (1979) Mechanisms of resistance to fenarimol in *Aspergillus nidulans*. Pestic Biochem Physiol 10: 219–229

De Waard M, Van Nistelrooy J (1980) An energy-dependent efflux mechanism for fenarimol in a wild-type strain and fenarimol-resistant mutants of *Aspergillus nidulans*. Pestic Biochem Physiol 13: 255–266

De Waard MA, Van Nistelrooy JGM (1987) Inhibitors of energy dependent efflux of the fungicide fenarimol by *Aspergillus nidulans*. Exp Mycol 11: 1–10

Decottignies A, Goffeau A (1997) Complete inventory of the yeast ABC proteins. Nature Genet 15: 137–145

Decottignies A, Grant AM, Nichols JW, deWet H, McIntosh DB, Goffeau A (1998) ATPase and multidrug transport activities of the overexpressed yeast ABC protein Yor1p. J Biol Chem 273: 12612–12622

Del Sorbo G, Andrade AC, Van Nistelrooy JGM, Van Kan JAL, Balzi E, De Waard MA (1997) Multidrug resistance in *Aspergillus nidulans* involves novel ATP-binding cassette transporters. Mol Gen Genet 254: 417–426

Doige CA, Yu X, Sharom FJ (1993) The effects of lipids and detergents on ATPase-active P-glycoprotein. Biochim Biophys Acta 1146: 65–72

Fernandes M, Keller NP, Adams TH (1998) Sequence-specific binding by *Aspergillus nidulans* AflR, a C-6 zinc cluster protein regulating mycotoxin biosynthesis. Mol Microbiol 28: 1355–1365

Fernandez E, Lombo F, Mendez C, Salas JA (1996) An ABC transporter is essential for resistance to the antitumor agent mithramycin in the producer *Streptomyces argillaceus*. Mol Gen Genet 251: 692–698

Fuchs HWM, Van der Lubbe JLM, Fuchs A (1981) The effect of cold storage, plant age and pod size on the ability of pea pods to accumulate pisatin. Acta Bot Neerland 30: 250

Gottesman MM, Pastan I (1993) Biochemistry of multidrug resistance mediated by the multidrug transporter. Annu Rev Biochem 62: 385–427

Guilfoile PG, Hutchinson CR (1991) A bacterial analog of the *mdr* gene of mammalian tumor cells is present in *Streptomyces peucetius*, the producer of daunorubicin and doxorubicin. Proc Natl Acad Sci USA 88: 8553–8557

Higgins CF (1992). ABC transporters: from microorganisms to man. Annu Rev Cell Biol 8: 67–113

Hirata D, Yano K, Miyahara K, Miyakawa T (1994) *Saccharomyces cerevisiae* YDR1, which encodes a member of the ATP-binding cassette (ABC) superfamily, is required for multidrug resistance. Curr Genet 26: 285–294

Hofmann K, Stoffel W (1993) TMbase – a database of membrane spanning proteins segments. Biol Chem Hoppe Seyler 374: 166

Hung LW, Wang IXY, Nikaido K, Liu PQ, Ames GFL, Kim SH (1998) Crystal structure of the ATP-binding subunit of an ABC transporter. Nature 396: 703–707

Kamp D, Haest CWM (1998) Evidence for a role of the multidrug resistance protein (MRP) in the outward translocation of NBD-phospholipids in the erythrocyte membrane. Biochim Biophys Acta 1372: 91–101

Kolaczkowski M, van der Rest M, Cybularz Kolaczkowska A, Soumillion JP, Konings WN, Goffeau A (1996) Anticancer drugs, ionophoric peptides, and steroids as substrates of the yeast multidrug transporter Pdr5p. J Biol Chem 271: 31543–31548

Kolaczkowski M, Kolaczkowska A, Luczynski J, Witek S, Goffeau A (1998) In vivo characterization of the drug resistance profile of the major ABC transporters and other components of the yeast pleiotropic drug resistance network. Microb Drug Resist 4: 143–158

Kuchler K, Sterne RE, Thorner J (1989) *Saccharomyces cerevisiae* STE6 gene product: a novel pathway for protein export in eukaryotic cells. EMBO J 8: 3973–3984

Leskela S, Wahlstrom E, Hyyrylainen HL, Jacobs M, Palva A, Sarvas M, Kontinen VP (1999) Ecs, an ABC transporter of *Bacillus subtilis*: dual signal transduction functions affecting expression of secreted proteins as well as their secretion. Mol Microbiol 31: 533–543

Lloyd AT, Sharp PM (1991) Codon usage in *Aspergillus nidulans*. Mol Gen Genet 230: 288–294

Loe DW, Stewart RK, Massey TE, Deeley RG, Cole SP (1997) ATP-dependent transport of aflatoxin B1 and its glutathione conjugates by the product of the multidrug resistance protein (MRP) gene. Mol Pharmacol 51: 1034–1041

Logemann J, Schell J, Willmitzer L (1987) Improved method for the isolation of RNA from plant tissues. Anal Biochem 163: 16–20

Mahe Y, Lemoine Y, Kuchler K (1996) The ATP binding cassette transporters Pdr5 and Snq2 of *Saccharomyces cerevisiae* can mediate transport of steroids in vivo. J Biol Chem 271: 25167–25172

Marger MD, Saier MH Jr (1993) A major superfamily of transmembrane facilitators that catalyse uniport, symport and antiport. Trends Biochem Sci 18: 13–20

Nagao K, Taguchi Y, Arioka M, Kadokura H, Takatsuki A, Yoda K, Yamasaki M (1995) *bfr1+*, a novel gene of *Schizosaccharomyces pombe* which confers brefeldin a resistance, is structurally related to the ATP-binding cassette superfamily. J Bacteriol 177: 1536–1543

Nakaune R, Adachi K, Nawata O, Tomiyama M, Akutsu K, Hibi T (1998) A novel ATP-binding cassette transporter involved in multidrug resistance in the phytopathogenic fungus *Penicillium digitatum*. Appl Environ Microbiol 64: 3983–3988

Neyfakh AA (1997) Natural functions of bacterial multidrug transporters. Trends Microbiol 5: 309–313

Nishi K, Yoshida M, Nishimura M, Nishikawa M, Nishiyama M, Horinouchi S, Beppu T (1992) A leptomycin B resistance gene of *Schizosaccharomyces pombe* encodes a protein similar to the mammalian P-glycoproteins. Mol Microbiol 6: 761–769

Penalva MA, Rowlands RT, Turner G (1998) The optimization of penicillin biosynthesis in fungi. Trends Biotechnol 16: 483–489

Pontecorvo G, Roper J, Hemmons L, MacDonalds K, Bufton A (1953) The Genetics of *Aspergillus nidulans*. Adv Genet 5: 141–238

Prasad R, De Wergifosse P, Goffeau A, Balzi E (1995) Molecular cloning and characterization of a novel gene of *Candida albicans*, CDR1, conferring multiple resistance to drugs and antifungals. Curr Genet 27: 320–329

Raeder U, Broda P (1985) Rapid preparation of DNA from filamentous fungi. Lett Appl Microbiol 1: 17–20

Raymond M, Gros P, Whiteway M, Thomas DY (1992) Functional complementation of yeast *ste6* by a mammalian multidrug resistance *mdr* gene. Science 256: 232–234

Romsicki Y, Sharom FJ (1998) The ATPase and ATP-binding functions of P-glycoprotein – Modulation by interaction with defined phospholipids. Eur J Biochem 256: 170–178

Ruetz S, Raymond M, Gros P (1993) Functional expression of P-glycoprotein encoded by the mouse *mdr3* gene in yeast cells. Proc Natl Acad Sci USA 90: 11588–11592

Sanger F, Nicklen S, Coulson AR (1977) DNA sequencing with chain-terminating inhibitors. Proc Natl Acad Sci USA 74: 5463–5467

Sanglard D, Ischer F, Monod M, Bille J (1996) Susceptibilities of *Candida albicans* multidrug transporter mutants to various antifungal agents and other metabolic inhibitors. Antimicrob Agents Chemother 40: 2300–2305

Sanglard D, Ischer F, Monod M, Bille J (1997) Cloning of *Candida albicans* genes conferring resistance to azole antifungal agents: characterization of CDR2, a new multidrug ABC transporter gene. Microbiology 143: 405–416

Seelig A (1998). A general pattern for substrate recognition by P-glycoprotein. Eur J Biochem 251: 252–261

Sharom FJ (1997) The P-glycoprotein multidrug transporter: interactions with membrane lipids, and their modulation of activity. Biochem Soc Trans 25: 1088–1096

Snedecor GW, Cochran WG (1989) Statistical methods (8th edn). Iowa State University Press, Ames

Thompson JD, Higgins DG, Gibson TJ (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 22: 4673–4680

Tobin MB, Peery RB, Skatrud PL (1997) Genes encoding multiple drug resistance-like proteins in *Aspergillus fumigatus* and *Aspergillus flavus*. Gene 200: 11–23

Urban M, Bhargava T, Hamer JE (1999) An ATP-driven efflux pump is a novel pathogenicity factor in rice blast disease. EMBO J 18: 512–521

Van Heemst D, Swart K, Holub EF, Van Dijk R, Offenberg HH, Goosen T, Van den Broek HWJ, Heyting C (1997) Cloning, sequencing, disruption and phenotypic analysis of *uvsC*, an *Aspergillus nidulans* homologue of yeast RAD51. Mol Gen Genet 254: 654–664

Van Helvoort A, Smith AJ, Sprong H, Fritzsche I, Schinkel AH, Borst P, Van Meer G (1996) MDR1 P-glycoprotein is a lipid translocase of broad specificity, while MDR3 P-glycoprotein specifically translocates phosphatidylcholine. Cell 87: 507–517

Van Tuyl J (1977) Genetics of fungal resistance to systemic fungicides. Mededelingen Landbouwhogeschool Wageningen 77: 1–136

Van Veen HW, Konings WN (1998) The ABC family of multidrug transporters in microorganisms. Biochim Biophys Acta 1365: 31–36

Van Veen HW, Callaghan R, Soceneantu L, Sardini A, Konings WN, Higgins CF (1998) A bacterial antibiotic-resistance gene that complements the human multidrug-resistance P-glycoprotein gene. Nature 391: 291–295

Volkman SK, Cowman AF, Wirth DF (1995) Functional complementation of the *ste6* gene of *Saccharomyces cerevisiae* with the *pfmdr1* gene of *Plasmodium falciparum*. Proc Natl Acad Sci USA 92: 8921–8925

Walker JE, Saraste M, Runswick MJ, Gay NJ (1982) Distantly related sequences in the alpha- and beta-subunits of ATP synthase, myosin, kinases and other ATP-requiring enzymes and a common nucleotide binding fold. EMBO J 1: 945–951

Wernars K, Goosen T, Wennekes BM, Visser J, Bos CJ, van den Broek HW, van Goorcom RF, van den Hondel CA, Pouwels PW (1985) Gene amplification in *Aspergillus nidulans* by transformation with vectors containing the *amdS* gene. Curr Genet 9: 361–368