Role of the ABC transporter TruMDR2 in terbinafine, 4-nitroquinoline N-oxide and ethidium bromide susceptibility in *Trichophyton rubrum*

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A single-copy gene, designated *TruMDR2*, encoding an ATP-binding cassette (ABC) transporter was cloned and sequenced from the dermatophyte *Trichophyton rubrum*. The ORF of *TruMDR2* was 4048 nt and the deduced amino acid sequence showed high homology with ABC transporters involved in drug efflux in other fungi. The encoded ABC protein predicted 12 transmembrane segments (TMSs) and two almost identical nucleotide-binding domains (NBDs) arranged in two halves in a (TMS6–NBD)2 configuration and could be classified as a member of the multidrug-resistance (MDR) class of ABC transporters. Northern blot analyses revealed an increased level of transcription of the *TruMDR2* gene when mycelium was exposed to acriflavine, benomyl, ethidium bromide, ketoconazole, chloramphenicol, griseofulvin, fluconazole, imazalil, itraconazole, methotrexate, 4-nitroquinoline N-oxide (4NQO) or tioconazole. Disruption of the *TruMDR2* gene rendered the mutant more sensitive to terbinafine, 4NQO and ethidium bromide than the control strain, suggesting that this transporter plays a role in modulating drug susceptibility in *T. rubrum*.

**INTRODUCTION**

*Trichophyton rubrum* is a cosmopolitan filamentous fungus that can infect human keratinized tissue (skin, nails and, rarely, hair) and is the causal agent of 80–90% of all chronic and recurrent dermatophytoses (Fernández-Torres et al., 2000). This pathogen, which normally causes well-characterized superficial infections, can also cause deep dermal invasion in immunocompromised patients (Smith et al., 2001; Squeo et al., 1998). Griseofulvin, terbinafine and itraconazole are widely used to treat dermatophytosis. Although in routine clinical practice the response to these antifungals is usually satisfactory, the occurrence of strains that are insensitive to antifungal agents (Mukherjee et al., 2003; Osborne et al., 2005) could play a relevant role in the failure of antifungal treatments. Thus, studies of the mechanisms of antifungal resistance are crucial for a more rational use of drugs, to minimize or overcome resistance.

A clinical isolate of *T. rubrum* submitted to a mutagenic treatment presented simultaneous resistance to griseofulvin and tioconazole *in vitro*, suggesting the existence of a multidrug-resistance (MDR) mechanism based on cellular efflux involved in this event (Fachin et al., 1996). The *T. rubrum* *TruMDR1* gene, which encodes an ATP-binding cassette (ABC) transporter, is also differentially expressed in the presence of unrelated toxic compounds, including the antifungals griseofulvin and itraconazole (Cervelatti et al., 2006). ABC transporters are highly conserved ATPases, ubiquitous from bacteria to humans, and this mechanism protects cells against the cytotoxic effects of compounds by reducing the accumulation of toxic compounds in the cell. In eukaryotes, most ABC transporters are composed of two similar halves, each consisting of a cytoplasmic nucleotide-binding domain (NBD) and six transmembrane segments (TMSs). The NBDs of ABC transporters contain conserved amino acid sequences, called the Walker A and Walker B motifs (Walker et al., 1982), and the ABC signature (Ames et al., 1990). Based on their topology, the ABC transporters can be subdivided into subfamilies or clusters. The best-characterized among these are those involved in MDR [[TMS6–NBD]2] or in pleiotropic drug resistance [[NBD–TMS6]2] (Wolfgang et al., 2001). ABC transporters have been described in several fungi and yeasts (De Hertogh et al., 2002; Del Sorbo et al., 2000) and may also be required for phytoalexin tolerance and virulence in phytopathogenic fungi such as *Botrytis cinerea* (Schoonbeek et al., 2001; Vermeulen et al., 2001), *Gibberella pulicaris* (Fleissner et al., 2002), *Magnaporthe grisea* (Urban et al., 1999) and

**Abbreviations:** ABC, ATP-binding cassette; MDR, multidrug resistance; NBD, nucleotide-binding domains; 4NQO, 4-nitroquinoline N-oxide; TMS, transmembrane segment.

The GenBank/EMBL/DDBJ accession number for the *TruMDR2* sequence of *Trichophyton rubrum* is AF291822.
Mycosphaerella graminicola (Stergiopoulos et al., 2002) and for secretion of endogenous secondary metabolites and xenobiotics in Aspergillus nidulans (Andrade et al., 2000). However, the role of ABC transporters in dermatophytes is still poorly understood.

In this paper, we report on the isolation, molecular cloning and functional analysis of TruMDR2, an ABC transporter-encoding gene of T. rubrum, which may be involved in modulating susceptibility to terbinafine, 4-nitroquinoline N-oxide (4NQO) and ethidium bromide. Furthermore, we describe the standardization of a transformation system in T. rubrum that was used for disruption of the TruMDR2 gene.

METHODS

Strains and growth conditions. The T. rubrum clinical isolate H6 (ATCC MYA-3108) was used throughout this study was cultured as described previously (Fachin et al., 1996, 2001). Escherichia coli strain LEM392 was used for amplification of the plasmid and strain DH5α was used for transformations and plasmid amplification. All transformants were selected on Luria broth medium (LB) containing ampicillin (50 μg ml⁻¹) and cultured at 37 °C overnight. The plasmid pCSN43, used for gene disruption, contained a gene encoding hygromycin phosphotransferase (hph) under the control of the promoter and terminator of the A. nidulans trpC gene (Fachin et al., 1996, 2001; Staben et al., 1989).

Construction of genomic libraries. DNA of the H6 strain was extracted from mycelia harvested after 72 h of incubation on liquid Sabouraud glucose medium at 28 °C and partially digested with SāaGAl. The fragments were sized by electrophoresis on a 0-7% low-melting-point agarose gel and purified from the gel. For construction of the plasmid library, vector pUC18 BanHI/BAP (Amersham Biosciences) was used to clone fragments of approximately 3–6 kb. For the lambda library, λGEM11 (Promega) was used to clone fragments of approximately 15–23 kb. The bacterial library had 3 × 10⁴ clones and the plasmid library had 10⁵ p.f.u.

Gene cloning and sequencing. A fragment of the TruMDR2 gene was cloned by PCR amplification using degenerate primers and T. rubrum H6 genomic DNA as template. Primers 5′-GGMTGSG-TGGYCCMTCGGG-3′ and 5′-AVGCRGADGTRCCYTCRTCTC-3′ were designed to the conserved amino acid sequences of the Walker motifs in ATP-binding domains of presumptive eukaryotic ABC-type transporters. Approximately 200 ng H6 isolate DNA, extracted as described by Raeder & Broda (1985), was used as template. PCR involved 30 cycles of denaturation at 94 °C, annealing at 60 °C and extension at 72 °C. The amplified products were resolved by electrophoresis on a 1% agarose gel. A sharp band of 380 bp was recovered from the gel, cloned into the pGEM-T vector (Promega), sequenced and used as a probe for screening of libraries. Hybridizing clones from phages were digested and subcloned into the pUC18 plasmid vector. The complete sequence of the TruMDR2 gene was determined by using the PHRED/PHRAP/CONSED package. The copy number of TruMDR2 in the genome of the H6 strain was determined by Southern blot analysis. Gene-specific probes were prepared and genomic DNA was digested with restriction enzymes Apal or SpeI, which do not cleave the TruMDR2 gene. The Random Primer DNA Labelling System (Invitrogen) was used to generate radiolabelled oligonucleotide probes with [α-32P]dCTP. DNA or clone transfer to membranes, hybridization, plasmid DNA preparation, phage DNA purification and digestion with restriction enzymes were carried out using standard protocols (Sambrook et al., 1989).

RNA extraction and Northern blot analysis. Conidia from T. rubrum were incubated in liquid Sabouraud glucose medium for 72 h at 28 °C with shaking at 160 r.p.m. and the resulting mycelia were harvested aseptically and transferred to fresh Sabouraud medium to which different drugs were added. They were incubated for 15 min (based on previous time-course experiments) under the same conditions, harvested by filtration and total mycelial RNA from each treatment was extracted with the Wizard RNA Isolation System (Promega). Approximately 20 μg RNA was electrophoresed on a 1% agarose gel containing formaldehyde, transferred to a Hybond-N⁺ membrane and hybridized with an internal fragment of the TruMDR2 gene radioactively labelled with [α-32P]dCTP, as described previously (Sambrook et al., 1989).

Initially, the susceptibility of T. rubrum to toxic agents was determined by an agar dilution assay on Sabouraud plates (Cervelatti et al., 2006). One hundred microlitres of a conidial suspension of approximately 5 × 10⁷ conidia ml⁻¹ was inoculated into Sabouraud glucose agar plates with various concentrations of the drugs and incubated at 28 °C for 7 days. As a control, the conidial suspension was inoculated into fungicide-free medium with the respective solvent. The subinhibitory concentrations used in Northern blot analysis, which corresponded to the concentrations of the drug at which there was approximately 90% inhibition of macroscopic growth (Cervelatti et al., 2006), were as follows: acriflavine, 2-5 μg ml⁻¹; benomyl, 2-5 μg ml⁻¹; etidium bromide, 2-5 μg ml⁻¹; ketoconazole, 10 μg ml⁻¹; cycloheximide, 30 mg ml⁻¹; chloramphenicol, 50 mg ml⁻¹; fluconazole, 300 μg ml⁻¹; griseofulvin, 2-0 μg ml⁻¹; imazalil, 4-0 μg ml⁻¹; irataconazole, 30 μg ml⁻¹; 4NQO, 10 μg ml⁻¹; terbinafine, 0-1 μg ml⁻¹; and tioconazole, 0-5 μg ml⁻¹. Benomyl was provided by Du Pont De Nemours. Imazalil, ketoconazole and itraconazole were provided by Jansen Pharmaceuticals. Cycloheximide was purchased from ICN Biomedicals. Fluconazole, terbinafine and chloramphenicol were provided by the University Hospital of Ribeirão Preto, São Paulo, Brazil. Tioconazole was provided by Pfizer. All other chemicals tested were purchased from Sigma. Acriflavine, etidium bromide, chloramphenicol and terbinafine were dissolved in sterile water. Benomyl, 4NQO, ketoconazole, itraconazole, tioconazole, imazalil, cycloheximide and griseofulvin were dissolved in DMSO. The final concentration of DMSO never exceeded 1% (v/v).

Disruption of the TruMDR2 gene. The Xbal–ClaI 2-9 kb fragment obtained from the D7P clone, which contains the TruMDR2 gene, was cloned into the unique Smal site of plasmid pUC18, to generate plasmid pXC (see Fig. 3). This plasmid, digested with EcoRV to promote a 135 bp deletion in the TruMDR2 gene, was used to clone the 2-4 kb Sal fragment hygromycin-resistance gene cassette (hph), filling in 5′ overhangs with Klenow polymerase (Amarach Biosciences), from pCSN43 to generate plasmid pXC (see Fig. 3). This plasmid, digested with EcoRV to promote a 135 bp deletion in the TruMDR2 gene, was used to clone the 2-4 kb Sal fragment hygromycin-resistance gene cassette (hph), filling in 5′ overhangs with Klenow polymerase (Amarach Biosciences), from pCSN43 to generate plasmid pXC, which was used without further modification to transform the H6 strain.

Protoplasts were obtained by incubating the mycelium harvested from two Petri dishes in 30 ml lytic solution consisting of 600 mg Glucanex (Novo Nordisk), 0-7 M KCl and 50 mM phosphate buffer (pH 5-8) for approximately 3–4 h at 28 °C. The protoplasts were filtered through glass wool, collected by centrifugation (5 min, 4 °C, 1556 g), washed with 10 ml CT buffer [0-4 M ammonium sulfate, 1% sucrose (w/v), 0-05 M citric acid (pH 6-0)] and collected by centrifugation. They were then washed with 10 ml DT buffer [0-6 M KCl, 0-1 M CaCl₂, 0-01 M Tris/HCl, pH 7-5], collected by centrifugation and the pellet was resuspended in 1 ml DT buffer and transformed with polyethylene glycol, as described by May (1992), using 10 μg plasmid pΔTruMDR2.
Transformants were selected in solid minimal medium (Cove, 1966) supplemented with 1 M sucrose containing 600 μg hygromycin ml⁻¹ at 28 °C. After 15 days, hygromycin-resistant colonies were clearly visible. These colonies appeared at a rate of 1:7 (μg plasmid)⁻¹ and (10⁷ protoplasts)⁻¹. No such colonies were detected when controls lacking DNA were plated on selective medium.

**Toxicant sensitivity assays.** Susceptibility of the H6 and ΔTruMDR2 mutant strains to cytotoxic agents was tested by assessing the MICs of the drugs according to the M38-A microdilution technique proposed by the National Committee for Clinical Laboratory Standards (NCCLS, 2002). Briefly, colonies obtained by growing the strains on Sabouraud plates at 28 °C for 15 days were covered with sterile saline containing 1% Tween 80. conidia were harvested by sterile scraping and the solution was filtered through glass wool. The resulting mixture of mostly non-germinated conidia was transferred to a sterile tube and adjusted spectrophotometrically at a wavelength of 530 nm ranging from 70 to 75% transmittance. These conidial suspensions were diluted 1:50 in RPMI 1640 (Sigma) buffered with MOPS, which corresponded to twice the density needed for the test of approximately 3 x 10⁻⁵–5 x 10⁻⁶ c.f.u. ml⁻¹. Each microdilution well containing 100 μl of the diluted (twice) drug concentrations was inoculated with 100 μl of the diluted (twice) conidial inoculum suspensions (final volume in each well was 200 μl). Growth and sterility controls were included for each isolate tested. Microtitre trays were incubated at 28 °C and MICs were recorded after 7 days of incubation. The susceptibility end point was recorded for each strain and for each drug. MIC₁₀₀ was defined as the lowest drug concentration resulting in total inhibition of visible growth.

**RESULTS AND DISCUSSION**

**Isolation and organization of the TruMDR2 gene**

We used degenerate primers based on the sequence of known MDR genes from a variety of organisms to amplify a 380 bp DNA fragment from *T. rubrum*. Cloning and DNA sequence analysis revealed that the amplified fragment encoded a highly conserved amino acid sequence characteristic of proteins containing an ABC. This PCR product was used to screen a genomic DNA plasmid library and led to the identification of the complete sequence of a gene named *TruMDR2* that contained an ORF of 4048 nt interrupted from nt 384 to 435 by a putative intron. Analysis of the deduced 1331 aa sequence of the encoded protein, TruMDR2, suggested the presence of 12 TMSs and two almost identical NBDs. These domains were arranged in two homologous halves in a (TMSₙ–NBD₂)₂ configuration, as predicted by the program PREDICT PROTEIN (Rost et al., 1995). An ABC was present in the N- and C-terminal halves of TruMDR2. The cassette in the hydrophilic moiety of both halves contained almost identical degenerate Walker A motifs (GsGxGxGK) with the lysine residue (K), which is highly conserved among members of the MDR transporters (Andrade et al., 2000; Angermayr et al., 1999; Tobin et al., 1997). In addition, the conserved glutamic acid residue (E) (Cutting et al., 1990) was present next to the Walker B motif in both halves of TruMDR2 (Fig. 1). The *T. rubrum* protein TruMDR2, which belongs to the subfamily 3.A.1 (transporter classification system), was 68% identical to AFUMDR1 of *Aspergillus fumigatus* (Tobin et al., 1997), 66% identical to AtrD of *A. nidulans* (Andrade et al., 2000), 63% identical to ABC4 of *Ventura inaequalis* (AAL57243) and 57% identical to AFLMDR1 of *Aspergillus flavus* (Tobin et al., 1997). These results suggest a close evolutionary relationship among these proteins. The putative protein TruMDR2 had an estimated molecular mass of 145 kDa and an isoelectric point of 6.7. Southern blot analysis of restriction enzyme-digested genomic DNA indicated that the *TruMDR2* gene is present as a single-copy gene (data not shown).

**Transcription of the TruMDR2 gene is enhanced by cytotoxic agents**

To verify the possible involvement of the *TruMDR2* gene in drug transport, the level of transcription of this gene was investigated following treatment of H6 mycelia with antifungal agents and some drugs known as substrates for ABC transporters from other organisms. Northern blot analysis showed that the *TruMDR2* gene was transcribed constitutively at low levels, was enhanced after 15 min of exposure to acriflavine, benomyl, ethidium bromide, ketoconazole, griseofulvin, fluconazole, imazalil, methotrexate and tioconazole, and was highly induced by chloramphenicol, itraconazole and 4NQO, compared with the untreated

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**Fig. 1.** Multiple alignment of the *T. rubrum* ABC TruMDR2 (TR) with representative members of the ABC transporter family. AN, *A. nidulans* (GenBank no. AAD43626); AF, *A. fumigatus* (GenBank no. AAB88658); VI, *Ventura inaequalis* (GenBank no. AAL57243); AF1, *Aspergillus flavus* (GenBank no. AAB88656). Identical residues are marked with asterisks. Dots and colons indicate conservative substitutions.
control (Fig. 2). These results indicated that TruMDR2 is easily induced by several chemically unrelated compounds. Semighini et al. (2002) observed that the ABC transporter encoded by the atrD gene of A. nidulans was induced in the presence of imazalil, itraconazole and 4NQO, showing that the TruMDR2 and atrD genes have several common substrates. On the other hand, cycloheximide induces the expression of the A. nidulans atrD gene (Andrade et al., 2000), but this induction was not observed for the TruMDR2 gene (Fig. 2), even though both of these microorganisms display intrinsic resistance to this compound (Angermayr et al., 1999; Weitzman & Summerbell, 1995). These results show the relevance of functional analysis of each ABC transporter, mainly in pathogenic fungi, as the proteins have an intrinsic property in each organism, as well as the fact that differences in the lipid composition of membranes may also affect substrate specificity (Andrade et al., 2000).

Disruption of the TruMDR2 gene

For functional analysis of the TruMDR2 gene, a disruptor cassette was constructed and inserted into the H6 strain by protoplast transformation, creating a TruMDR2 deletion allele (ΔTruMDR2) by one-step gene replacement. We replaced 135 bp of the ORF of TruMDR2 with a gene encoding hygromycin phosphotransferase (hph) to form the gene-replacement vector pΔTruMDR2 (Fig. 3a). Transformants were selected on hygromycin-containing medium and the TruMDR2 deletion was confirmed by Southern blot analysis. As expected, genomic DNA of the H6 and ΔTruMDR2 strains digested with EcoRI and SacI restriction enzymes and probed with the P-380 fragment (Fig. 3a) showed a hybridization signal at 694 bp for the H6 strain, whereas the mutant showed a hybridization signal at 2954 bp, which was the length expected for a correct gene-replacement event (Fig. 3b).

Northern blot analysis was carried out with total RNA isolated from mycelia of the ΔTruMDR2 mutant treated
with itraconazole, a strong inducer of TruMDR2 transcription, and probed with the fragment excised from the TruMDR2 gene (P-135). No hybridization was observed, confirming that TruMDR2 had been functionally deleted (data not shown). The ΔTruMDR2 mutant was viable and grew in Sabouraud medium as well as the control strain H6.

Thus, the DNA transformation method developed for Neurospora crassa (Staben et al., 1989) to disrupt or replace genes by homologous recombination events was successfully used here for T. rubrum, permitting, in the future, the functional study of other genes of this fungus. It is also noteworthy that the standardization of DNA transformation and disruption systems for T. rubrum amplifies the repertoire of molecular techniques for the study of this dermatophyte.

### Sensitivity of the ΔTruMDR2 mutant to toxicants

The susceptibility of the T. rubrum strains to the various inhibitory agents was also determined by estimating their MICs in RPMI 1640. Table 1 shows that the ΔTruMDR2 mutant displayed increased sensitivity to terbinafine, 4NQO and ethidium bromide when compared with the H6 strain. In contrast, there was no difference in the level of sensitivity between the ΔTruMDR2 mutant and the control strain when submitted to the other drugs tested. However, in spite of our results suggesting that the TruMDR2 gene modulates terbinafine susceptibility, Northern blot analysis revealed that the level of transcription of this gene, when T. rubrum was cultured in the presence of terbinafine, was at the same basal level as that observed in untreated cultures (Fig. 2).

It is likely that the terbinafine concentration used in the Northern blot analysis (0·1 μg ml⁻¹) was not sufficient to activate transcription of the TruMDR2 gene, suggesting that the fungus has another mechanism to deal with terbinafine at low concentrations. In A. nidulans, it has been shown that terbinafine resistance can be mediated through the overexpression of salA, a gene that encodes a salicylate 1-monooxygenase. This enzyme is involved in naphthalene degradation, suggesting that resistance could follow degradation of the naphthalene ring contained in terbinafine (Graminha et al., 2004).

Ethidium bromide and 4NQO are substrates for some multidrug-resistance pumps. Increased expression of two multidrug transporter-like genes is associated with ethidium bromide and ciprofloxacin resistance in Mycoplasma hominis (Raherison et al., 2002, 2005). 4NQO, a potent carcinogen, is activated metabolically to a form that reacts with DNA. In Saccharomyces cerevisiae, total deletion of the SNQ1 gene (ABC transporter) was shown to increase the sensitivity of the mutants to 4NQO (Gömpel-Klein & Brendel, 1990).

We cannot rule out the possibility of the TruMDR2 gene playing a role by modulating the susceptibility to the other drugs that increase the levels of TruMDR2 transcripts, as observed by Northern blot analysis. However, the presence of additional forms of ABC transporter proteins in T. rubrum may compensate for the deletion of the TruMDR2 gene by acting as an efflux pump to provide resistance to these toxic compounds. This could be the case for itraconazole, an antifungal agent used to control dermatophytosis, which strongly induced transcription of TruMDR2 and to which the ΔTruMDR2 mutant strain presented no change in sensitivity at the itraconazole concentrations assayed (several concentrations were assayed; data not shown). In fact, the T. rubrum TruMDR1 gene is also induced by many of these drugs, including itraconazole (Cervelatti et al., 2006), in spite of TruMDR1 showing only 29% identity with TruMDR2. In Candida albicans, increased sensitivity to some antifungals could be observed only when a double-deletion strain, for genes CDR1 and CDR2 (ABC transporters), was constructed, because Cdr1p was able to compensate for the lack of Cdr2p (Sanglard et al., 1997).

In conclusion, the growth phenotype observed for the ΔTruMDR2 mutant suggests that the TruMDR2 gene, although present as a single copy, is not essential for the in vitro survival of T. rubrum. We presume that the TruMDR2 transporter-mediated toxicant efflux mechanism plays a role in modulating susceptibility to terbinafine, 4NQO and ethidium bromide in T. rubrum. A study of the molecular mechanisms of T. rubrum resistance/susceptibility is attractive because this organism is an important

### Table 1. Susceptibilities of the T. rubrum H6 and ΔTruMDR2 strains to cytotoxic compounds

MIC₁₀₀ corresponded to the lowest concentration of the toxic compound at which there was no macroscopic growth. The results are representative of two independent experiments performed in triplicate.

<table>
<thead>
<tr>
<th>Antifungal and cytotoxic agents</th>
<th>MIC₁₀₀ (μg ml⁻¹)</th>
<th>H6 (wild-type)</th>
<th>ΔTruMDR2</th>
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<tr>
<td>Acriflavine</td>
<td>1·25</td>
<td>1·25</td>
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<tr>
<td>Benomyl</td>
<td>0·37</td>
<td>0·37</td>
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<tr>
<td>Ethidium bromide</td>
<td>1·25</td>
<td>0·75</td>
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<td>0·06</td>
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<td>37·5</td>
<td></td>
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<td>37·5</td>
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<tr>
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<tr>
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</tr>
<tr>
<td>Hygromycin*</td>
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<td>&gt;6000</td>
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</table>

*Hygromycin was used to select the hygromycin-resistant colonies after transformation with the plasmid pΔTruMDR2.
human pathogen and is becoming a model for biomedical research on dermatophytes.

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