Membrane transporter proteins are involved in \textit{Trichophyton rubrum} pathogenesis

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\textit{Trichophyton rubrum} is a dermatophyte responsible for the majority of human superficial mycoses. The functional expression of proteins important for the initial step and the maintenance of the infection process were identified previously in \textit{T. rubrum} by subtraction suppression hybridization after growth in the presence of keratin. In this study, sequences similar to genes encoding the multidrug-resistance ATP-binding cassette (ABC) transporter, copper ATPase, the major facilitator superfamily and a permease were isolated, and used in Northern blots to monitor the expression of the genes, which were upregulated in the presence of keratin. A sequence identical to the \textit{TruMDR2} gene, encoding an ABC transporter in \textit{T. rubrum}, was isolated in these experiments, and examination of a \textit{T. rubrum} \textit{TruMDR2} mutant showed a reduction in infecting activity, characterized by low growth on human nails compared with the wild-type strain. The high expression levels of transporter genes by \textit{T. rubrum} in mimetic infection and the reduction in virulence of the \textit{TruMDR2} mutant in a disease model \textit{in vitro} suggest that transporters are involved in \textit{T. rubrum} pathogenicity.

INTRODUCTION

Fungal infections can cause morbidity in immunosuppressed individuals, particularly those undergoing transplants and chemotherapy, and human immunodeficiency virus-positive patients, showing the importance of early diagnosis and appropriate antifungal therapy (Erbagci, 2002; Nir-Paz \textit{et al.}, 2003). The dermatophytes – fungi specialized in keratin degradation – are frequently involved in chronic infections that affect humans on a global scale. The most prevalent dermatophyte is \textit{Trichophyton rubrum}, which has been isolated from tinea corporis, tinea unguium and tinea pedis infections (Summerbell, 1997; Weitzman & Summerbell, 1995).

Several studies have provided evidence that a pH environment-mediated effect regulates fungal gene expression and secretory activity (Casadevall & Pirofski, 2001; Ferreira-Nozawa \textit{et al.}, 2006), both of which play a role in pathogenicity. During keratin degradation, dermatophytes have been shown to secrete specific enzymes, depending on sensing of the host ambient pH (Maranhão \textit{et al.}, 2007; Martinez-Rossi \textit{et al.}, 2004). A regulatory sensing system in the response of \textit{T. rubrum} towards carbon-source availability and the ability to adapt in the host is essential for the survival of this fungus (Ferreira-Nozawa \textit{et al.}, 2006; Kunert, 1972, 1976), inducing the expression of enzymes and membrane transporters that facilitate the uptake of amino acids (Grobler \textit{et al.}, 1995; Lechenne \textit{et al.}, 2007). Furthermore, specific genes need to be expressed to enable these mechanisms, which are involved in the activation of signal transduction for maintenance of the infection process under keratin contact. The occurrence of therapeutic failures due to the development of resistance is often associated with efflux pumps in the cell membrane (Martinez-Rossi \textit{et al.}, 2008; Prasad \textit{et al.}, 2006). Recent observations have indicated that membrane transporters are commonly involved in bacterial and fungal pathogenesis, with important roles as virulence factors to ensure successful colonization in a host environment (Mishra \textit{et al.}, 2007). The \textit{TruMDR1} gene of \textit{T. rubrum} encodes an ATP-binding cassette (ABC) transporter and is expressed differentially in the presence of antifungals such as griseofulvin and itraconazole (Cervelatti \textit{et al.}, 2006). Early genetic knockout analysis of a \textit{TruMDR2} strain has shown that a \textit{TruMDR2} transporter-mediated toxicant efflux mechanism plays a role in modulating susceptibility to terbinafine, 4-nitroquinoline N-oxide and ethidium bromide in \textit{T. rubrum} (Fachin \textit{et al.}, 2006). The aim of this study was to investigate the differential expression of genes.
encoding proteins similar to transporters in the dermatophyte *T. rubrum* grown in medium containing keratin, and to compare growth characteristics between a wild-type and a Δ*TruMDR2* strain during nail infection. Our findings suggest that membrane transporter proteins are important in the dermatophyte infection process, adding new knowledge about the gene expression profile of *T. rubrum*.

**METHODS**

**Fungal strains and culture conditions for expression analyses.** The *T. rubrum* H6 (wild-type) strain (ATCC MYA-3108) and strain Δ*TruMDR2* (Fachin et al., 2006) were used throughout this study and were cultured as described previously (Fachin et al., 1996). In expression assays, *T. rubrum* wild-type conidia were grown in liquid minimal medium with nitrate (70 mM) as the nitrogen source (Cove, 1966), supplemented with keratin in the test sample (2.5 g ml⁻¹) to promote specific gene expression, or with glucose in the control sample (55 mM), for 72 h at 28 °C at an initial pH of 5.0.

**Isolation of RNA, cDNA synthesis and construction of suppression subtractive hybridization (SSH) library.** Total RNA from the wild-type *T. rubrum* strain was extracted from approximately 100 mg frozen mycelium using TRIzol reagent (Invitrogen). The quality and concentration of total RNA were checked by formaldehyde/agarose gel electrophoresis and a spectrophotometer. A 1 µg sample was used for double-stranded cDNA synthesis using a BD SMART PCR cDNA synthesis kit (BD Biosciences Clontech), according to the manufacturer’s guidelines. An SSH library was constructed as described by Diatchenko et al. (1996) and Maranha˜o et al. (2007) using a PCR Select cDNA subtraction kit (BD Biosciences Clontech). We used *T. rubrum* mycelia treated in the presence of keratin as the tester population and in the presence of glucose as the driver population. The final PCR sample obtained corresponded to genes differently expressed when this dermatophyte was in contact with keratin. All PCR products were cloned into the pGEM-T Easy Vector system (Promega) and transformed into Escherichia coli (Mos Blue). After selection by X-Gal/IPTG, white colonies were picked and grown in Luria–Bertani medium with selection by ampicillin and X-Gal/Blue. After selection by X-Gal/IPTG, white colonies were picked and grown in Luria–Bertani medium with selection by ampicillin and tetracycline resistance for 20 h at 37 °C, and stored until needed for the next steps.

**DNA sequencing and Northern blotting.** The cDNA clones were sequenced on an ABI Prism 377 DNA sequencer (Applied Biosystems), using M13 forward and reverse primers. The sequences were processed using the PhredPhrap-Consed package and submitted for similarity searches against the non-redundant GenBank database using BLASTX software at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov) (Altschul et al., 1997). Clones with inserts corresponding to transporter genes were selected for use as probes in Northern blotting analysis.

For validation of differential gene expression by Northern blotting, *T. rubrum* was cultivated under test and control conditions (keratin and glucose, respectively). Total RNA (15 µg) from *T. rubrum* grown under each condition was separated by electrophoresis on a 1.5% agarose/formaldehyde gel and subsequently transferred to nylon membranes (Hybond-N⁺; Amersham) using a vacuum blotter (Bio-Dot; Bio-Rad). Probes were purified from plasmids containing inserts of the specific transporter genes by PCR amplification. All probes (50 ng) were labelled by random priming (Random Primers DNA labelling system; Gibco-BRL) and [α-32P]dCTP. Hybridization was performed overnight at 65 °C with agitation, and membranes were washed using standard protocols (Sambrook et al., 1989) and exposed to storage phosphor screens (Applied Biosystems). The intensity level of each transcript was quantified based on the number of digital light units generated using OptiQuant (Perkin Elmer) after subtracting the background. The Northern blotting results shown are representative of two independent experiments.

**Infection assays.** Infection *in vitro* was performed as described by Takasuka (2000). Human nail fragments of approximately 1 × 1 mm were treated with ethanol for 15 min and dried at room temperature. A 5 µl aliquot of a conidial suspension (3 × 10⁷ ml⁻¹) of each *T. rubrum* strain (wild-type and mutant) was soaked onto individual nail fragments for 1 h, followed by the addition of 200 µl distilled water in Eppendorf tubes. The tubes were incubated at 28 °C for 6 days, and fungal growth was observed by light microscopy (Axion Vision system; Zeiss). Nail fragments soaked in 200 µl distilled water were used as a control.

**RESULTS AND DISCUSSION**

**Identification of transporter genes by SSH and bioinformatic analyses**

In this study, we investigated the expression of several genes that may affect *T. rubrum* pathogenesis. After the isolation of genes differentially expressed by SSH, which used *T. rubrum* mycelium cultivated in keratin as the test condition, a BLASTX analysis was performed and 66 clones similar to genes encoding putative transporters from different families were identified. These represented 27.7% of the cDNA clones isolated after *T. rubrum* was grown in the presence of keratin (Maranha˜o et al., 2007). From the 66 subtractive cDNA clones screened, 5 non-redundant unique expressed sequence tags (ESTs) were generated. These ESTs, named TR0042, TR0043, TR0044, TR0052 and TR0097 (GenBank accession nos EB086877, EB086878, EB086879, EB086887 and EH038250), were predicted to encode proteins corresponding to the major facilitator superfamily (MFS) peptide transporter, copper resistance-associated P-type ATPase, multidrug-resistance (MDR) transporter, amino acid permease and V-type ATPase, respectively (Table 1). Most of these proteins show similarity to well-known homologues in *Aspergillus fumigatus*. MFS proteins, members of a large transporter family, occurred at a high incidence (57.6%) among the transporter proteins expressed in *T. rubrum* grown in the presence of keratin (Table 1).

It is known that the human skin has a pH of approximately 5.0 (Marro et al., 2001) and contains keratinized elements, which are important factors for dermatophyte infection. Thus, our experiments were performed at an initial pH of 5.0, with keratin or glucose as the carbon source. The results were obtained after 72 h of *T. rubrum* cultivation (when the keratin medium reached pH 8.4), an experimental condition appropriate for the isolation of genes likely to be involved in the maintenance of infection, which are preferentially expressed at alkaline pH (Maranha˜o et al., 2007).

**Expression of transporter genes in *T. rubrum***

Northern blot analysis was carried out with total RNA isolated from the mycelia of wild-type *T. rubrum* H6 strain...
cultivated with keratin or glucose. The mRNA steady-state levels of genes encoding specific efflux pumps, a metal transporter, the MFS and a permease were detected by Northern blotting analysis under test conditions, during germination in keratin-containing medium, revealing that the expression of specific transporters in this test medium was higher than that in glucose-containing medium (Fig. 1). The ABC and MFS transporters are two major classes of proteins involved in drug resistance. The substrate range of both membrane transporters is very broad and may include ions, amino acids, peptides, sugars, secondary metabolites and drugs. Several studies have investigated the role of the MDR transporter in response to metabolic poisons and antifungal drugs (Prasad et al., 2006). BLASTX analysis revealed the identity of a sequence that was highly upregulated in the test cDNA population (TR0044, GenBank accession no. EB086879) with a T. rubrum MDR-like ABC transporter, a multidrug pump efflux (Fachin et al., 2006). Fungal ABC transporters play a function as efflux pumps, providing resistance to several antifungal types (Mishra et al., 2007; Urban et al., 1999). In addition, ABC-encoding genes have been identified as an important pathogenicity determinant in the early stages of filamentous fungi pathogenesis, as demonstrated for the ABC1 from Magnaporthe grisea, BcatrB from Botrytis cinerea and GpABC1 from Gibberella pulicaris. (Fleissner et al., 2002; Gupta & Chattoo, 2008; Vermeulen et al., 2001).

Additionally, the Northern blotting analyses with a probe similar to the MFS transporter-encoding gene showed a strong signal under the test keratin conditions (Fig. 1).

**Table 1.** *T. rubrum* transporter genes overexpressed during growth in the presence of keratin

<table>
<thead>
<tr>
<th>EST</th>
<th>GenBank accession no.</th>
<th>Size (bp)</th>
<th>Putative ID (GenBank protein no.)</th>
<th>E value</th>
<th>Identity (%)</th>
<th>No. of clones</th>
</tr>
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<tr>
<td>TR0042</td>
<td>EB086877</td>
<td>634</td>
<td><em>A. fumigatus</em>, MFS peptide transporter, putative, XP_746951</td>
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<td>TR0097</td>
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<td>224</td>
<td><em>A. fumigatus</em>, V-type ATPase, subunit B, XP_755966</td>
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<td>16</td>
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<td>TR0044</td>
<td>EB086879</td>
<td>420</td>
<td><em>T. rubrum</em>, MDR protein, AAG01549 (TruMDR2 gene)</td>
<td>2e−20</td>
<td>100</td>
<td>9</td>
</tr>
<tr>
<td>TR0043</td>
<td>EB086878</td>
<td>176</td>
<td><em>A. fumigatus</em>, copper resistance-associated P-type ATPase, XP_754347</td>
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<td>2</td>
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<tr>
<td>TR0052</td>
<td>EB086887</td>
<td>723</td>
<td><em>A. fumigatus</em>, amino acid permease, putative acid permease, AAC98709</td>
<td>8e−12</td>
<td>57</td>
<td>1</td>
</tr>
</tbody>
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**Fig. 1.** Northern blotting analyses showing differential expression of *T. rubrum* genes encoding an MFS transporter (TR0042), a copper (Cu²⁺) ATPase (TR0043), an ABC MDR transporter (TR0044) and a permease (TR0052), using total RNA from wild-type strain H6. This strain was cultivated in glucose as a control (C) and in keratin (K). Ethidium bromide-stained rRNA shows the relative loading of the samples. The graphs indicate the fold differences in the intensity of the Northern blots shown by each clone expressed in the H6 strain cultivated under control and test conditions, as established by densitometry analysis using a PhosphorImager.
Although a large number of clones similar to MFS-encoding gene were isolated by SSH when *T. rubrum* was grown in keratin-containing medium, we also detected a weak hybridization signal under control conditions in comparison with the test sample. This result was expected because MFS transporters are a ubiquitous group of proteins involved in the transport of a wide range of compounds, including external elements and toxins (Saier et al., 1999; Vardy et al., 2004). In the *Asperillus oryzae* genome, 63.9% of transporters are MFS type, involved in secondary metabolism (Akao et al., 2007). The analysis of a gene encoding MFS transporter-like protein (CTB4) in *Cercospora nicotianae* provided genetic evidence to support its role in virulence by cercosporin accumulation (Choquer et al., 2007). In this study, experiments with a *Cercospora nicotianae* mutant displayed a drastic reduction in cercosporin production and accumulation, causing fewer lesions on tobacco leaves (Choquer et al., 2007).

Expression of the copper ATPase-encoding sequence was just detectable as an approximately 3 kb transcript in the wild-type strain grown in minimal medium containing keratin. A subtractive library enriched by *T. rubrum* genes expressed in the presence of cytotoxic components showed the upregulation of a gene encoding copper-resistance-associated P-type ATPase after fluconazole contact (Paia˜o et al., 2007). Copper is an essential metabolic element as a cofactor of many cellular enzymes, and copper-transporting P-type ATPases are found in eukaryotic organisms and require an iron transporter for adaptation in different environments and hosts (Riggle & Kumamoto, 2000). The absence of normal copper-transporter activity in *Cryptococcus neoformans* after VPH1 gene deletion was observed in an avirulent mutant interfering in laccase activity (Zhu & Williamson, 2003), a strong virulence factor in this opportunistic pathogen. In addition, a disruption in the *Candida albicans* CaCCC2 gene led to an unusual phenotype of silver and copper resistance (Weissman et al., 2002), and a *clap1* mutation in *Colletotrichum lindemuthianum* caused a non-pathogenic phenotype (Parisot et al., 2002), both genes encoding a putative copper-transporting ATPase.

The virulence capacity was also affected in *Listeria monocytogenes* mutants, carrying a mutation in *cptA*, a gene involved in copper homeostasis (Francis & Thomas, 1997). Furthermore, our Northern blot experiments also demonstrated that a permease, a general transporter, was highly expressed in *T. rubrum* cultures growing in the test medium.

**Assay of infection by ΔTruMDR2**

The effect of disrupting the *TruMDR2* gene (TR0044 cDNA) on the pathogenicity of *T. rubrum* was assessed in the nail model *in vitro*, to test the infection ability of the ΔTruMDR2 strain mutant in comparison with wild-type H6. Conidia preparations of wild-type and mutant strains were distributed separately on sterile nails, and growth was observed by microscopy 6 days after inoculation and incubation at 28 °C. The deletion of *TruMDR2* was found to correlate with a decreased ability of the ΔTruMDR2 mutant to grow on human nail (Fig. 2), a major infection site of *T. rubrum*.

The *TruMDR2* gene, encoding an ABC MDR transporter in *T. rubrum*, is also highly expressed after exposure to acrilavine, ketoconazole, chloramphenicol, griseofulvin, fluconazole and other cytotoxic agents, as revealed by Northern blotting of the wild-type strain. Also, the mutant ΔTruMDR2 is more sensitive to terbinafine, 4-nitroquinoline N-oxide and ethidium bromide than the control strain, confirming the involvement of this gene in drug transport (Fachin et al., 2006). The MDR genes may affect other aspects of metabolism and nutrient availability, and, in our *T. rubrum* infection assays in nails, the mutant phenotype of the ΔTruMDR2 strain was found to correlate strictly with decreased growth on human nails.

In conclusion, the capacity of infecting fungi to regulate their gene expression and overcome host resistance is crucial in the virulence of dermatophytes. The expression of several transporters reported in our studies confirmed that a membrane transporter system is required for optimal growth on keratin sources and may reflect a regulatory role in the establishment of dermatophytoses at the host site.

**Fig. 2.** Infection assays showing the growth of different *T. rubrum* strains cultivated on human nail for 6 days at 28 °C. The fungal growth on human nails was observed by light microscopy and the images were captured using an Axio Vision system (Zeiss) with a Plan Neofluar 20× 0.5 objective. The black regions on the left side of the images are nail fragments: (a) negative control (no inoculum); (b) H6 (wild-type) strain; (c) ΔTruMDR2 strain. Representative results of two experiments are shown.
Furthermore, by using nail fragments as the only source of nutrients, we found that the ΔTruMDR2 mutant grew poorly compared with the control wild-type strain, suggesting that the TruMDR2 gene may be involved in the virulence of *T. rubrum*.

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