Sulphite efflux pumps in Aspergillus fumigatus and dermatophytes

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Dermatophytes and other filamentous fungi excrete sulphite as a reducing agent during keratin degradation. In the presence of sulphite, cystine in keratin is directly cleaved to cysteine and S-sulphocysteine, and thereby, reduced proteins become accessible to hydrolysis by a variety of secreted endo- and exoproteases. A gene encoding a sulphite transporter in Aspergillus fumigatus (AfuSsu1), and orthologues in the dermatophytes Trichophyton rubrum and Arthrodema benhamiae (TruSsu1 and AbeSsu1, respectively), were identified by functional expression in Saccharomyces cerevisiae. Like the S. cerevisiae sulphite efflux pump Ssu1p, AfuSsu1p, TruSsu1p and AbeSsu1p belong to the tellurite-resistance/dicarboxylate transporter (TDT) family which includes the Escherichia coli tellurite transporter TehAp and the Schizosaccharomyces pombe maltose transporter Mae1p. Seven genes in the A. fumigatus genome encode transporters of the TDT family. However, gene disruption of AfuSsu1 and of the two more closely related paralogues revealed that only AfuSsu1 encodes a sulphite efflux pump. TruSulp and AbeSulp are believed to be the first members of the TDT family identified in dermatophytes. The relatively high expression of TruSsu1 and AbeSsu1 in dermatophytes compared to that of AfuSsu1 in A. fumigatus likely reflects a property of dermatophytes which renders these fungi pathogenic. Sulphite transporters could be a new target for antifungal drugs in dermatology, since proteolytic digestion of hard keratin would not be possible without prior reduction of disulphide bridges.

INTRODUCTION

The dermatophytes are a group of common closely related fungi which are responsible for the great majority of human superficial mycoses (Kwong-Chung & Bennet, 1992; Weitzman & Summerbell, 1995). These highly specialized pathogenic fungi are almost exclusively found in the epidermal stratum corneum, hair or nails. The battery of secreted proteases secreted by dermatophytes is similar to that of Aspergillus, but differs by multiple endoprotease members of the S8 (subtilisins) and M36 (fungalysins) family (see MEROPS proteolytic enzyme database; http://merops.sanger.ac.uk) (Jousson et al., 2004a, b). Although it is evident that the secreted proteolytic activity of these fungi is important for degradation of compact keratinous tissues, fungal secreted proteases are incapable by themselves of dissolving structures made of hard keratin which are rich in cystine. Efficient keratin degradation by hydrolytic enzymes has to be accompanied by the simultaneous reduction of cystine disulphide bridges, which are mainly responsible for the resistant nature of keratin (Kunert, 1992, 2000). Moreover, efficient degradation in vitro of the hair structure of keratin azure by hydrolytic enzymes is only possible in the presence of a reducing agent, e.g. 1 % β-mercaptoethanol or DTT (Jousson et al., 2004b).

During keratin degradation, dermatophytes and filamentous fungi have been shown to excrete sulphite as a reducing agent (Kunert, 1972a, 1976). In the presence of sulphite, disulphide bonds of the keratin substrate are directly cleaved to cysteine and S-sulphocysteine. The presence of sulphocysteine has been confirmed by histochemical methods in human hair attacked by Microsporum gypseum (Kunert, 1972b). This compound was found in the free form and in oligopeptides of ~700–2500 Da which were products of keratinolysis (Kunert, 1976; Ruffin et al., 1976). As highly
specialized fungus, the dermatophytes are able to metabolize free cystine added to a nutrient broth, and excrete excess sulphur as sulphate and sulphite. Because sulphite is immediately consumed by sulphitolyis of sulhide peroxidases, this compound is only detectable when its amount exceeds that of cystine (Kunert, 1975, 2000).

The necessity to reduce disulphide bridges in the digestion of cornified tissues instigated our investigation of the mechanism of sulphite secretion in dermatophytes. In dermatophytes, as well as in the opportunistic mould *Aspergillus fumigatus*, we have identified genes encoding sulphite efflux pumps by heterologous expression in *Saccharomyces cerevisiae*, which results in a sulphite-resistant phenotype. Sulphite transporters in keratinolytic fungi could be a new target for antifungal drugs in dermatology.

METHODS

**Strains and plasmids.** Trichophyton rubrum CHUV1673-05, Arthroderma benhamiae CBS112371 (Fumeaux et al., 2004) and *A. fumigatus* D141 (NRRL 6585, US Department of Agriculture, Peoria, IL) were used in this study. All plasmid subcloning experiments were performed in *Escherichia coli* XL-1 Blue using plasmids pMTL21 (Chambers et al., 1988), pUC18 and pCL1920. The diploid *S. cerevisiae* strain INVSc1 (MATa, his3D1, leu2, trpl-289, ura3-52; MATa, his3D1, leu2, trpl-289, ura3-52) and the expression vector pYES2 (Invitrogen) were used to express heterologous transporters.

**Growth media.** *T. rubrum*, *A. benhamiae* and *A. fumigatus* were grown on Sabouraud agar and liquid medium (Bio-Rad), or in soy protein liquid medium (SP) and keratin liquid medium (KSP) (Monod et al., 2005) to promote production of proteolytic activity (KSP described as). Cystine–arginine liquid medium (CAM) [0.8 % (w/v) glucose, 0.2 % (w/v) arginine, 0.04 % (w/v) KH2PO4, 3 mM (0.072 %, w/v) cystine] (Kunert, 1982) was used to demonstrate secretion of sulphite. Cystine was added to the sterile medium in the form of a 10 × concentrated solution in HCl (1 M) and the medium was neutralized with KOH. A volume of 100 ml medium was inoculated with a plug of freshly growing mycelium in 800 ml tissue-culture flasks. The cultures were incubated for 10 days at 30 °C without shaking.

Complete medium for *S. cerevisiae* was YEPD [2 % (w/v) Difco Bacto peptone, 1 % (w/v) Difco Bacto yeast extract, 2 % (w/v) glucose]. *S. cerevisiae* synthetic minimal medium supplemented with histidine, leucine and tryptophan (20 μg ml−1) was prepared according to Sherman (1991). For expression of genes cloned in pYES2 under the control of the GAL promoter, galactose was added instead of glucose as the carbon source. YEPD made Na2SO3 stock solution (0.25 or 0.5 M) onto agar plates containing 25 ml medium, and allowing the sulphite to dry and diffuse overnight at room temperature.

**Sulphite and S-sulphocysteine analysis.** Supernatants of cultures in SP and KSP media were first treated by Pronase (10 μg ml−1) (Roche Diagnostics) and porcine kidney aminopeptidase-M (2 U ml−1) (Calbiochem) for 24 h at 37 °C. Free S-sulphocysteine was determined after deproteinization treatment of the sample with 32 % (w/v) sulphosalicylic acid. Amino acid analysis was performed by HPLC with a fluorescence detector, after precolumn derivatization with O-phthalaldehyde-3-mercaptopropionate and 9-fluorenylmethyl-chloroformate (FMOC) (Henderson et al., 2000). The concentration of free sulphite was determined by the fuchsin-formaldehyde method (Scaringelli et al., 1967).

**Expression of tellurite-resistance/dicarboxylate transporters (TDFs) in *S. cerevisiae*.** cDNAs encoding the transporters *AfusSsu1p*, *AfusSsul1p*, *AfusSsul2p*, *TrusSsu1p* and *AbesSsu1p* were obtained by RT-PCR using a OneStep RT-PCR kit (Qiagen). Briefly, 20 ng total RNA, 10 μl of the supplied 5× OneStep RT-PCR buffer (12.5 mM MgCl2, pH 8.7), 2 μl deoxynucleotide mix containing 10 mM each dNTP, 5 μl sense and antisense primers (P1–P8, Table 1) at a concentration of 6 μM, and 2 μl OneStep RT-PCR enzyme mix were mixed on ice, and subsequently incubated at 50 °C for 30 min and 95 °C for 15 min. The reaction mixtures were then subjected to 35 cycles of 0.5 min at 94 °C, 0.5 min at 55 °C and 1 min at 72 °C, and finally incubated for 10 min at 72 °C. Alternatively, a cDNA library of *A. fumigatus* D141 (Denikus et al., 2005) was used to amplify cDNAs encoding *A. fumigatus* transporters. PCR was performed with the homologous primers P1–P6 (Table 1) and 200 ng of DNA prepared from 107 clones of the cDNA library, using a standard protocol (Jousson et al., 2004a, b).

A DNA fragment encoding SceSsu1p was amplified by PCR using the primers P9 and P10 (Table 1), and *S. cerevisiae* INVSc1 genomic DNA as a target. For amplification, a standard protocol was used (Jousson et al., 2004a, b).

Expression plasmids were constructed by cloning PCR products in the *S. cerevisiae* expression vector pYES2. The PCR products were purified with a PCR purification kit (Roche Diagnostics) and then digested with restriction enzymes for which a site was previously designed at the 5′ end of the primers. The cloned fragments were further sequenced and the absence of possible PCR-induced errors was confirmed. Plasmid DNA was prepared from one *E. coli* clone harbouring a correct construct. *S. cerevisiae* transformations were performed using a transformation kit and according to the recommendations of the supplier (Invitrogen). Selection of URA3 transformants was performed using minimal medium with glucose and required amino acids.

Transformants were tested for sulphite resistance using low-pH medium, as described by Park et al. (1999). Yeasts were grown to the mid-exponential phase (OD600 1.0) at 30 °C in minimal medium with galactose and required amino acids. Each culture was diluted to OD600 1.0. Subsequently, 5 μl of serial dilutions (100–106) were spotted onto YEPL + TA plates containing the desired concentration of sulphite. The plates were incubated at 30 °C for 2 days.

**A. fumigatus gene disruption.** Gene disruption vectors were constructed using pAN7.1 (Punt et al., 1987) and 1.0 kb-sized internal fragments of the respective *A. fumigatus* gene. In detail, gene fragments were obtained by PCR using appropriate primers (P1–P16, Table 1) and genomic *A. fumigatus* DNA as the template. The PCR products were first cloned into pCR-Script Amp SK (+) (Stratagene). In a second step, the *A. fumigatus* fragments were excised from the plasmid constructs with *Stul* and *BstEI*, for which a site was introduced into the primers, and ligated to the larger fragment of pAN7.1 digested with the same restriction enzymes. The generated plasmids were termed pASSU1, pASSU1 and pASSU2. Undigested plasmids were used for subsequent gene-targeted disruption experiments.

Transformation of *A. fumigatus* D141 was performed according to a protocol that has been used for *Aspergillus nidulans* and *A. fumigatus* (Tilburn et al., 1983; Paris, 1994) with 107 protoplasts and 5 μg of plasmid. After overnight expression of the hygromycin B phosphotransferase gene (*HphI*), the transformants were incubated on agar plates containing 25 μl medium, and allowing the sulphite to dry and diffuse overnight at room temperature.
Table 1. Primers used in this study

<table>
<thead>
<tr>
<th>Primer</th>
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<th>Location</th>
<th>PCR product size with cloning sites</th>
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<td>P1</td>
<td>5'-GTTAAGCTCTACATGCTGACGCTGCAATACTAACAT-3'</td>
<td>AfuSSU1</td>
<td>1213 bp</td>
</tr>
<tr>
<td>P2</td>
<td>5'-CTTAAGCTCTACATGCTGACGCTGCAATACTAACAT-3'</td>
<td>Complement of AfuSSU1</td>
<td>HindIII/EcoRI</td>
</tr>
<tr>
<td>P3</td>
<td>5'-GTTACGCTCAACATGGAACTCCCTGCTCAGAATACAAAAT-3'</td>
<td>AfuSSUL1</td>
<td>1213 bp</td>
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<td>P4</td>
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<td>SstI/XbaI</td>
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<td>AfuSSUL2</td>
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<tr>
<td>P6</td>
<td>5'-GTTAAGCTCTACATGCTGACGCTGCAATACTAACAT-3'</td>
<td>Complement of AfuSSUL2</td>
<td>Asp718/XbaI</td>
</tr>
<tr>
<td>P7</td>
<td>5'-GTTAAGCTCTACATGCTGACGCTGCAATACTAACAT-3'</td>
<td>TraSU1/AbeSSU1</td>
<td>1153 bp/1153 bp</td>
</tr>
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<td>Complement of TraSU1/AbeSSU1</td>
<td>HindIII/EcoRI</td>
</tr>
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<td>BstEII/Stul</td>
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<td>Downstream sequence in AfuSSU1</td>
<td>1152 bp</td>
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<td>P24</td>
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<td>Upstream sequence in AfuSSU1</td>
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<td>P25</td>
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<td>Downstream sequence in AfuSSU1</td>
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<td>5'-GTTAAGCTCTACATGCTGACGCTGCAATACTAACAT-3'</td>
<td>Upstream sequence in AfuSSU1</td>
<td>1152 bp</td>
</tr>
</tbody>
</table>

Based on minimal medium (Cove, 1966) containing 200 μg hygromycin ml⁻¹ (Sigma), and selected after 5 days incubation at 20 °C followed by an overnight incubation at 42 °C. Transformants initially identified as hygromycin resistant were selected and subcultured again on agar containing hygromycin. Typically, 100–200 hygromycin-resistant colonies were obtained for each transformation using 10⁷ protoplasts and 5 μg plasmid DNA.

The A. fumigatus disruptants were identified by PCR of genomic DNA from various numbers of hygromycin-resistant colonies as a template and two pairs of specific primers (P17–P28, Table 1). Each primer pair was designed to yield a product of a predicted size when the respective plasmid had integrated at a homologous site. In each primer pair, one primer hybridized with the transformation plasmid and the other primer hybridized with genomic DNA near the desired homologous integration locus, as exemplarily shown for the AfuSSU1 disruption (Fig. 1). The primer pairs P17 to P20, P21 to P24 and P25 to P28 (Table 1) were used for screening sul1, sul1 and sul2 mutants, respectively.

Positive PCR screening of 20 hygromycin-resistant colonies in each experiment led to the identification of three, one and 10 disruption mutants for the genes encoding AfuSul1p, AfuSul1p and AfuSul2p, respectively. Neither the wild-type parental strain nor those transformants with presumed ectopically integrated plasmids showed a positive reaction.

**Sulphite resistance of A. fumigatus and dermatophytes.** A. fumigatus and dermatophytes were grown for 4 and 10 days, respectively, at 30 °C on Sabouraud medium. YEPA + TA medium containing the desired concentration of sulphite were inoculated with A. fumigatus conidia or dermatophyte mycelium. Alternatively, 100 ml liquid YEPA + TA medium containing sulphite was inoculated in 800 ml tissue-culture flasks. Dermatophyte and A. fumigatus cultures were incubated for 10 and 3 days, respectively, at 30 °C without shaking.

**T. rubrum and Arth. benhamiae gene cloning.** Recombinant plagues (2 x 10⁶) of a previously constructed T. rubrum ΔEMBL3 genomic DNA library were immobilized on GeneScreen nylon membranes (NEN Life Science Products). The filters were hybridized with a 3²P-labelled AfuSSU1 probe under low-stringency conditions (Monod et al., 1994). All positive plaques were purified, and the associated bacteriophage DNAs were isolated as described elsewhere (Grossberger, 1987). Agarose gel electrophoresis of restricted recombinant bacteriophage ΔEMBL3 DNA, Southern blotting and subcloning
of hybridizing fragments from bacteriophages into pMTL21 or pUC19 were performed using standard protocols (Sambrook et al., 1989). DNA sequencing was performed by Microsynth. T. rubrum and Arth. benhamiae cDNAs were obtained by RT-PCR using homologous primers designed from genomic DNA sequences.

**RNA preparations and Northern-blotting experiments.** The RNA of filamentous fungi was prepared from 10-day-old 100 ml liquid cultures in different media. The mycelium was ground in liquid nitrogen to a fine powder using a mortar and pestle, and the total RNA was isolated using an RNeasy Plant Mini Kit (Qiagen). Northern-blotting experiments were performed as described by Sambrook et al. (1989).

**RESULTS**

**Sulphite secreted by filamentous fungi**

*T. rubrum* and *Arth. benhamiae* grew well at 30 °C in a medium containing 0.2 % arginine and 0.07 % cystine as the only nitrogen sources. In contrast, germ tubes alone developed from *A. fumigatus* conidia in cystine–arginine liquid medium. After 20 days growth, a substantial amount of S-sulphocysteine was measured in the culture supernatants of the dermatophytes (0.7–2.0 m mol ml⁻¹). Comitantly, sulphite was detected at concentrations of 0–0.16 m mol ml⁻¹ and 0.64–0.80 m mol ml⁻¹ for *T. rubrum* and *Arth. benhamiae*, respectively. S-Sulphocysteine was also detected in the supernatants of the dermatophyte and *A. fumigatus* cultures in KSP protein medium at concentrations ranging from 0.01 to 0.035 m mol ml⁻¹, at which point substantial endoproteolytic activity was recorded and the media were totally clarified (15–20 days for dermatophytes, 30–40 days for *A. fumigatus*). Free sulphite was not detected in KSP protein medium. The MIC for sulphite measured in YEPD + TA medium was 3 mM for the dermatophytes and 1.5 mM for *A. fumigatus*.

**Cloning of genes encoding *A. fumigatus* and dermatophyte sulphite efflux pumps**

The high concentrations of S-sulphocysteine and sulphite in dermatophyte culture supernatants in cystine media led us to identify sulphite efflux pumps in these fungi. A sulphite efflux pump, Ssu1 (called in this paper SceSsu1p), has been characterized elsewhere in *S. cerevisiae* (Avram & Bakalinsky, 1997). Overexpression of the gene SceSsu1 enhances the resistance of *S. cerevisiae* to sulphite, which is used as a preservative agent in fermentations (Park et al., 1999; Park & Bakalinsky, 2000; Donalies & Stahl, 2002). SceSsu1p belongs to the TDT family, which includes the *E. coli* tellurite transporter and the Schizosaccharomyces pombe malate transporter encoded by the genes TEHA and MAEI, respectively (Walter et al., 1991; Grobler et al., 1995; see the TransportDB database, http://www.membranetransport.org/). Our strategy to find sulphite efflux pumps in dermatophytes consisted of trying to isolate the SceSsu1 orthologue in *A. fumigatus* for which the genome sequence is available. If a hit was obtained, the orthologues in dermatophyte genomes could subsequently be sought. Indeed, it is known that the nucleotide sequences of dermatophyte genes exhibit 50–70 % identity to the orthologous genes of *Aspergillus* spp. (Jousson et al., 2004a, b; Monod et al., 2005).

A BLASTP analysis with SceSsu1p on the *A. fumigatus* Af293 genome (www.tigr.org/tdb/e2k1/afu1) revealed seven genes encoding putative transporters of the TDT family. Three transporters with the highest similarity to SceSsu1p, corresponding to Afu7g01790, Afu3g14640 and Afu1g13360 in the TransportDB database, were retained for gene expression experiments in *S. cerevisiae* (Fig. 2). Three corresponding cDNAs could specifically be amplified using 5’-sense and 3’-antisense primers (P1–P6, Table 1) by RT-PCR of *A. fumigatus* D141 RNA, or by PCR based on DNA extraction from a pool of 10⁶ clones of a previously

**Fig. 1.** Plasmid construct and predicted outcome of the pΔSsu1 integration event. A 990 bp internal PCR fragment of *AfuSSU1* was cloned into the pAN7.1 plasmid (Punt et al., 1987). pAN7.1 carries the hygromycin resistance gene (HPH) from *E. coli* as a dominant selectable marker under the control of the GPD promoter (pGPD) and TRPC terminator (tTRPC) from *A. nidulans*. Homologous recombination of the plasmid construct with *AfuSSU1* by a single crossover event resulted in the generation of two incomplete copies of the *A. fumigatus* gene [labelled SSU1 (N-term) and SSU1 (C-term)] separated by the linearized sequence of pAN7.1. The primers used to screen for the incomplete *AfuSSU1* gene were P17 and P18 (5’ fragment) and P19 and P20 (3’ fragment). Further information on primers is in Table 1.

**Table 1.** Primers for screening for incomplete *AfuSSU1* gene.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Source</th>
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<tr>
<td>P17</td>
<td>TGGATGCTAGACAACTGAT</td>
<td>pAN7.1</td>
</tr>
<tr>
<td>P18</td>
<td>GTCGCTTCGTCGTCGTCG</td>
<td>pAN7.1</td>
</tr>
<tr>
<td>P19</td>
<td>TGGATGCTAGACAACTGAT</td>
<td>pAN7.1</td>
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<tr>
<td>P20</td>
<td>GTCGCTTCGTCGTCGTCG</td>
<td>pAN7.1</td>
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**Note:** The primers were designed to screen for the incomplete *AfuSSU1* gene. Further information on primers is in Table 1.
constructed cDNA library as the template. The nucleotide sequences of the obtained cDNA fragments were 100% identical to sequences in the *A. fumigatus* Af293 genome from which introns were removed. However, their deduced amino acid sequences revealed some differences from the deposited amino acid sequences of Afu7g01790, Afu3g14640 and Afu1g13360, respectively. The deduced amino acid sequences of AfuSsu1p, AfuSsu1p and AfuSsu2p revealed some differences from the deposited amino acid sequences for Afu3g14640, Afu1g13360 and Afu7g01790 in the TransportDB database. The branch lengths are proportional to the similarity between amino acid sequences. Amino acid sequence accession numbers at NCBI are as follows: SpoMae1, AAC49149; ScoSsu1p, NP_015233; TruSsu1p, DQ777768; AbeSsu1p, EF035480; AfuSsu1p, AAX54670; AfuSsu1p, AAX54671; AfuSsu2p, AAX54672; Afu8g04630, EAL85231; Afu4g04540, EAL84602; Afu4g09410, EAL89846; Afu2g17660, EAL94063; *E. coli* TehAp (EcoTehAp), AAA19563.

Fig. 2. Neighbour-joining (NJ) phylogenetic tree of TDT transporters, including seven members recorded in *A. fumigatus*. AfuSsu1p, AfuSsu1p and AfuSsu2p substitute Afu7g01790, Afu3g14640 and Afu1g13360, respectively. The deduced amino acid sequences of AfuSsu1p, AfuSsu1p and AfuSsu2p revealed some differences from the deposited amino acid sequences for Afu3g14640, Afu1g13360 and Afu7g01790 in the TransportDB database. The branch lengths are proportional to the similarity between amino acid sequences. Amino acid sequence accession numbers at NCBI are as follows: SpoMae1, AAC49149; ScoSsu1p, NP_015233; TruSsu1p, DQ777768; AbeSsu1p, EF035480; AfuSsu1p, AAX54670; AfuSsu1p, AAX54671; AfuSsu2p, AAX54672; Afu8g04630, EAL85231; Afu4g04540, EAL84602; Afu4g09410, EAL89846; Afu2g17660, EAL94063; *E. coli* TehAp (EcoTehAp), AAA19563.

Fig. 3. (A) Susceptibility and resistance to sulphite (1.6 mM) of *S. cerevisiae* transformed with pYES2 (top line) and with different plasmids encoding TDT transporters. *S. cerevisiae* was spotted at different dilutions on YEPG+TA plates, as described in Methods. (B) As a control, *S. cerevisiae* was spotted on plates without sulphite. The plates were incubated at 30 °C for 2 days.

AY861352). The two other genes were called *AfuSSUL1* and *AfuSSUL2*, encoding Ssu-like protein 1 and Ssu-like protein 2 in *A. fumigatus* (GenBank accession nos AY861353 and AY861354, respectively). *AfuSSU1*, *AfuSSUL1* and *AfuSSUL2* revealed similar collinear structures with four introns and five exons.

In parallel, *A. fumigatus* disruption mutants were constructed and tested for sulphite resistance, as described in Methods. *A. fumigatus ssu1* mutants were highly sensitive to sulphite (Fig. 4), while the resistance to sulphite of *ssul1* and *ssul2* mutants was comparable to that of the wild-type strain of the fungus. These results confirmed that only *AfuSSU1*

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Fig. 4. Susceptibility and resistance to sulphite of *A. fumigatus* D141 wild-type (wt) and mutated strains. YEPD+TA plates with 0.8 mM sulphite (A) and without sulphite (B) were spotted with 5 μl of a suspension of *A. fumigatus* conidia (10⁶ ml⁻¹). The plates were incubated at 30 °C for 2 days. YEPD+TA plates containing sulphite were prepared by spreading an appropriate amount of freshly made Na₂SO₃ stock solution onto agar plates containing 25 ml medium, and allowing the sulphite to dry and diffuse overnight at room temperature.
encodes a sulphite efflux pump among the three selected genes of *A. fumigatus* encoding transporters of the TDT family. However, the *A. fumigatus ssu1* mutants grew in a similar manner to the wild-type strain in SP and KSP media, and *S*-sulphocysteine was detected in the culture supernatant in KSP medium.

*AfuSSU1*, *AfuSSUL1* and *AfuSSUL2* were predicted to encode proteins of 396, 396 and 436 amino acid residues, with molecular masses of approximately 43.6, 44.0 and 47.6 kDa, respectively. Like the *Schiz. pombe* malate transporter encoded by the *MAEI* gene (Grobler et al., 1995), the deduced amino acid sequences revealed proteins with hydrophilic N and C termini and 10 putative membrane-spanning helices which are typical of membrane transport proteins (Fig. 5). The length of the hydrophilic linkers ranged from 3 to 20 amino acids.

A 1200 bp fragment containing the whole nucleotide sequence of *AfuSSU1* was used as a probe for screening a λEMBL3 phage *T. rubrum* genomic DNA library. All isolated hybridizing clones contained a nucleotide sequence encoding a gene 50% identical to *AfuSSU1*. The corresponding cDNA encoding a putative sulphite transporter could be specifically amplified by RT-PCR using 5′-sense and 3′-antisense primers (Table 1) and total RNA extracted from *T. rubrum* mycelium in cystine–arginine medium. An *Arth. benhamiae* cDNA encoding a putative transporter differing from that of *T. rubrum* by two amino acids (Val63 and Ala206 in *Arth. benhamiae* instead of Ile63 and Val206 in *T. rubrum*) was also amplified using the same primers. *S. cerevisiae* became resistant to 1.6 mM sulphite when these amplified cDNAs were expressed under the control of the GAL promoter (Fig. 3). The genes encoding these sulphite transporters in *T. rubrum* and *Arth. benhamiae* were called *TruSSU1* and *AbeSSU1* (GenBank accession nos DQ777768 and EF035480, respectively). *TruSSU1* and *AbeSSU1* revealed an intron–exon structure similar to that of *AfuSSU1*. Both *TruSSU1* and *AbeSSU1* were predicted to encode proteins of 375 amino acid residues with molecular masses of approximately 42 kDa, hydrophilic N and C termini, and 10 putative membrane-spanning helices (Fig. 5).

**Expression of sulphite transporter genes in *A. fumigatus* and dermatophytes**

Northern-blot analysis revealed that *TruSSU1* and *AbeSSU1* encode single transcripts of approximately 2000 bp each. Expression of *TruSSU1* and *AbeSSU1* in cystine–arginine and KSP media was higher than that in Sabouraud and SP media (Fig. 6). Although *AfuSSU1* DNA could be amplified by RT-PCR with RNA extracted from *A. fumigatus* grown in YEPD + TA containing 1.2 mM sulphite (300 mM below the MIC), SP and KSP media, no *AfuSSU1* signal was detected in comparably sensitive Northern blotting experiments and utilizing the whole *AfuSSU1* cDNA as a probe.

**DISCUSSION**

We present what is believed to be the first molecular genetic report on sulphite efflux pumps in dermatophytes and in filamentous fungi. The number of genes within the fungal genome that encode transporters of the TDT family to which these pumps belong varies from species to species. In *S. cerevisiae*, SceSsu1p is the only member of this family. In contrast, the TDT family has seven, six, three and two members in *A. fumigatus*, *Aspergillus oryzae*, *A. nidulans* and *Neurospora crassa*, respectively (see the TransportDB)

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**Fig. 5.** Transmembrane protein topology predicted using PRODIV-TMHMM (http://www.pdc.kth.se/~hakanv/prodiv-tmhmm/) (Viklund & Elofsson, 2004). The plot was obtained by calculating the total probability that a residue sits within a helix (box), inside (lowered line) or outside the protein (raised line), summed over all possible paths through the model. The two portions of the alignment shown in Fig. 7 are indicated by asterisks.
database). However, gene disruption of AfuSSU1 and of the 
two more closely related paralogues in A. fumigatus revealed 
that only AfuSSU1 encodes a sulphite efflux pump. The 
multitude of transporters of the TDT family in a saprophytic 
and opportunistic fungus such as A. fumigatus is likely to 
confer other selective advantages in terms of resistance to the 
different chemical compounds which are present in varied 
environments. TruSSu1p and AbeSSu1p are believed to be 
the first transporters of the TDT family to be described in 
dermatophytes. No further paralogues were identified from 
a screening of the T. rubrum genomic library performed with 
TruSSU1 as probe.

AfuSSu1p, TruSSu1p and AbeSSu1p are only 20% identical 
to SceSSu1p. In contrast, A. fumigatus transporters of the 
TDT family which are not sulphite efflux pumps are more 
similar to AfuSSu1p (Fig. 2). Alignments of TDT transport- 
ers included in Fig. 2 show that the identified sulphite efflux 
pumps have only two short conserved amino acid sequences 
(Fig. 7). Two potential signature sequences for sulphite 
transporters in the TDT family could be derived from the 
portions of the alignment shown in Fig. 7 as follows:

(1) GT(F/Y)PMG(F/L)XTIIN

(2) LP(I/L)GP(L/M)GQG(G/S)(F/Y)G

Sulphite is a product of the metabolism of cysteine 
compounds assimilated by fungi. Although a regular 
fungal metabolite, sulphite is potentially toxic in the 
eryplasm (Thomas & Surdin-Kerjan, 1997). Excess sulphite 
is generally excreted by fungi after oxidation in the form of 
inorganic sulphate (Obata & Ishikawa, 1959; Kunert, 1989).

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Fig. 6. Northern blots of T. rubrum, Arth. bennhamiae and A. fumigatus total RNA hybridized with cDNA fragments (pYES2 inserts) encoding the whole of TruSSu1p, AbeSSu1p and AfuSSu1p, respectively, as a probe. Mycelium was harvested from growing hyphae in the following liquid media: Sabouraud (A), SP (B), KSP (C), cystine-arginine (D) and YEPD+TA containing 1.2 mM sulphite (E). The variable number of days of each culture is indicated in the bottom line of the figure. In SP and KSP media, substantial endoproteolytic activity was recorded, and the media were totally clarified. In cystine-arginine medium, a substantial amount of S-sulphocysteine was measured in the culture supernatants. Approximately 4 μg total RNA was loaded in each lane. As an intensity standard, 4 μg total RNA from S. cerevisiae grown on synthetic minimal medium with galactose and expressing TruSSU1, AbeSSU1 and AfuSSU1 was loaded in parallel (lanes F). The position of the hybridizing bands obtained from S. cerevisiae transformants is lower, because only the parts of the cDNAs encoding TruSSu1p, AbeSSu1p and AfuSSU1p, respectively, were cloned in pYES2. 28S and 18S rRNAs are indicated to the right of the figure by < and ≪, respectively.

Fig. 7. Domains of homology between the deduced amino acid sequences of sulphite efflux pumps TruSSu1p, AbeSSu1p, AfuSSu1p and SceSSu1p, two other closely related members of the TDT family, AfuSSu1p and AbeSSu1p, and SpoMae1p (amino acid sequence accession numbers at NCBI in legend of Fig. 2). Numbers on the right indicate the amino acid positions in the aligned proteins. Dots represent identical amino acids.
Excretion of sulphite mediated by an efflux pump represents an alternative detoxification pathway for dermatophytes during infection of the epidermal stratum corneum, hair and nails, which are rich in cysteine. At the same time, the ability of dermatophytes to grow within hard keratin depends on the secretion of sulphite in order to reduce proteins. Once disulphide bridges are directly cleaved by sulphitolysis, reduced proteins become accessible to further digestion by various secreted endo- and exoproteases. Therefore, efflux-pump-mediated sulphite detoxification and sulphitolysis may be considered as complementary functions in the digestion of compact keratinous tissue.

The relatively high expression of TruSSU1 and AbeSSU1 compared to that of AfuSSU1 (Fig. 6) likely reflects a property of dermatophytes which renders these fungi pathogenic in the epidermal stratum corneum, hair and nails. Dermatophytes were able to grow more rapidly than A. fumigatus in KSP medium. Hard keratin grains were totally digested after 20 days under our culture conditions, while 40 days were necessary to observe clarification of the medium with A. fumigatus. Although sometimes isolated as a contaminant from nails, the latter fungus is not an aetiologic agent of infection in hard keratinous tissues. Apparently, A. fumigatus growth in keratin-containing medium does not depend on AfuSsu1 activity, since the A. fumigatus ssu1 mutants were able to grow well in KSP medium, and S-sulphocysteine was detected in the culture supernatant. Thus, the existence of another sulphite efflux pump cannot be excluded in A. fumigatus, but it is also possible that small amounts of sulphite can leave the mycelium by a route other than that of sulphite transporters.

No fewer than 16 genes encoding secreted endo- and exopeptidases have been found in dermatophytes (Jousson et al., 2004a,b; Monod et al., 2005). The hypothesis that one keratinase, alone or with further proteases, decomposes hard keratin, has now been abandoned (Kunert, 1992). Dermatophyte-secreted subtilisins have been shown to be at most 50% more active towards keratin azure than proteinase K or subtilisin Carlsberg, with the last two enzymes both in the presence of ß-mercaptoethanol (Jousson et al., 2004b). Sulphitolysis is so far the sole known dermatophyte mechanism that allows the reduction of disulphide bridges, a bottleneck in the process of degradation of compact keratinized tissues. In contrast, the activities of the dermatophyte-secreted proteases are redundant for the digestion of reduced protein (Jousson et al., 2004b; Monod et al., 2005). Therefore, sulphite transporters, rather than secreted proteases, could be a target of choice to treat dermatophyte infection. Given the absence of TDT transporters in humans, mechanism-based toxicity would most likely be minimal.

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