Sulphite efflux pumps in *Aspergillus fumigatus* and dermatophytes

Barbara Léchenne,¹ Utz Reichard,² Christophe Zaugg,¹ Marina Fratti,¹ Jiri Kunert,³ Olivier Boulat⁴ and Michel Monod¹

Correspondence

Michel Monod
Michel.Monod@chuv.ch

¹Department of Dermatology, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

²Department of Medical Microbiology and National Reference Center for Systemic Mycoses, University Hospital of Göttingen, Germany

³Department of Biology, Faculty of Medicine, Olomouc, Czech Republic

⁴Clinical Chemistry Laboratory, Centre Hospitalier Universitaire Vaudois, Lausanne, Switzerland

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 *Arthroderma 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* malate 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. *TruSsulp* and *AbeSsulp* 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.

Received 10 October 2006
Revised 23 November 2006
Accepted 7 December 2006

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 proteases secreted by dermatophytes is similar to that of *Aspergillus*, but differs by multiple endoprotease members of the S8 (subtilisins) and M36 (fungalsins) 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 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

Abbreviation: TDT, tellurite-resistance/dicarboxylate transporter.

The GenBank/EMBL/DDBJ accession numbers for the *AfuSSU1*, *AfuSSUL1*, *AfuSSUL2*, *TruSSU1* and *AbeSSU1* sequences reported in this paper are AY861352, AY861353, AY861354, DQ777768 and EF035480, respectively.

specialized fungi, 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 sulphitolysis of disulphide bridges, 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*, *trp1-289*, *ura3-52*; MAT α , *his3D1*, *leu2*, *trp1-289*, *ura3-52*) and the expression vector pYES2 (Invitrogen) were used to express heterologous transporters.

Growth media. *T. rubrum*, *Arth. 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, as previously described.

Cystine–arginine liquid medium (CAM) [0.8% (w/v) glucose, 0.2% (w/v) arginine, 0.04% (w/v) KH_2PO_4 , 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 $10\times$ concentrated solution in HCl (1 M) and the medium was neutralized with KOH. A volume of 100 ml liquid 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 ($20\text{ }\mu\text{g ml}^{-1}$) 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 + TA (YEPD containing 75 mM L-tartaric acid and buffered at pH 3.5) was prepared as described elsewhere (Park *et al.*, 1999). YEPG + TA was prepared in the same way as YEPD + TA with 2% galactose instead of glucose. YEPG + TA plates containing sulphite were prepared by spreading an appropriate amount of freshly made Na_2SO_3 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 ($10\text{ }\mu\text{g ml}^{-1}$) (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) sulphasalicylic acid. Amino acid analysis

was performed by HPLC with a fluorescence detector, after pre-column 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 (TDTs) in *S. cerevisiae*. cDNAs encoding the transporters AfuSsu1p, AfuSsu1p, AfuSsu2p, TruSsu1p and AbeSsu1p were obtained by RT-PCR using a OneStep RT-PCR kit (Qiagen). Briefly, 20 ng total RNA, 10 μl of the supplied $5\times$ OneStep RT-PCR buffer (12.5 mM MgCl_2 , 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 10^6 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 (OD_{600} 1.0) at 30 °C in minimal medium with galactose and required amino acids. Each culture was diluted to OD_{600} 1.0. Subsequently, 5 μl of serial dilutions (10^0 – 10^4) were spotted onto YEPG + 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 (P11–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 *StuI* and *BstEII*, 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 10^7 protoplasts and 5 μg of plasmid. After overnight expression of the hygromycin B phosphotransferase gene (*HPH*), the transformants were incubated on agar

Table 1. Primers used in this study

Nucleotides in italic type represent cloning sites.

Primer	Oligonucleotide sequence	Location	PCR product size with cloning sites
P1	5'-GTTAAGCTTCAACATGAGCTGCGGCAATACAAAT-3'	<i>AfuSSU1</i>	1213 bp
P2	5'-CTTGAATTCTCAAGCAGCCTTCGCAACGTC-3'	Complement of <i>AfuSSU1</i>	<i>HindIII/EcoRI</i>
P3	5'-GTTGAGCTCCAACATGGAACCCCGTCTCAAACCA-3'	<i>AfuSSUL1</i>	1213 bp
P4	5'-CTTTCTAGATCATGCTTGGCGAATTGGAGC-3'	Complement of <i>AfuSSUL1</i>	<i>SacI/XbaI</i>
P5	5'-GTTGGTACCCAACATGAGCACGACTACCAAGCCTTGT-3'	<i>AfuSSUL2</i>	1333 bp
P6	5'-CTTTCTAGATCAAGATTGTGATATGCTTTCC-3'	Complement of <i>AfuSSUL2</i>	<i>Asp718/XbaI</i>
P7	5'-GTTAAGCTTCAACATGCCATCAGGATCCGGCTTCCATAA-CATCG-3'	<i>TruSSU1/AbeSSU1</i>	1153 bp/1153 bp
P8	5'-CTTGAATTCTCATCGGAAGAGCTCCTTGGTGCATGA-3'	Complement of <i>TruSSU1/AbeSSU11</i>	<i>HindIII/EcoRI</i>
P9	5'-GTTGAGCTCCAACATGGTTGCCAATTGGGTACTT-3'	<i>SceSSU1</i>	1399 bp
P10	5'-CTTGAATTCTTATGCTAAACGCGTAAATC-3'	Complement of <i>SceSSU1</i>	<i>XhoI/EcoRI</i>
P11	5'-TAGGTGACCTGATGTGGGCTGGAGGAGGATC-3'	<i>AfuSSU1</i>	990 bp
P12	5'-TAAGGCCTCCGAAGGACCAGAGATGAGCG-3'	Complement of <i>AfuSSU1</i>	<i>BstEII/StuI</i>
P13	5'-TAGGTGACCTCGCGTAGTCAGCCACTTCAGC-3'	<i>AfuSSUL1</i>	990 bp
P14	5'-TAAGGCCTAGTCGCCAGGGCAAATACAAG-3'	Complement of <i>AfuSSUL1</i>	<i>BstEII/StuI</i>
P15	5'-TAGGTGACCACTGGCTGGAGACGAATCGTG-3'	<i>AfuSSUL2</i>	990 bp
P16	5'-TAAGGCCTCTACGACATAAACTATGGAGGCG-3'	Complement of <i>AfuSSUL2</i>	<i>BstEII/StuI</i>
P17	5'-TCCATCAGATAATGAGCTGCGG-3'	Upstream sequence in <i>AfuSSU1</i>	1153 bp
P18	5'-GAATATCCTCTTGACACCGCTCC-3'	pAN7.1 sequence	
P19	5'-GGTTAAGATCCACGTCTTCGGG-3'	pAN7.1 sequence	1069 bp
P20	5'-GAGCGATAGAAGCTGACGCG-3'	Downstream sequence in <i>AfuSSU1</i>	
P21	5'-AATGGAACCCCGTCTCAAACC-3'	Upstream sequence in <i>AfuSSUL1</i>	1152 bp
P22	5'-TATCCTCTTGACACCGCTCCG-3'	pAN7.1 sequence	
P23	5'-GTCGATCATCCGCTGAAGGC-3'	pAN7.1 sequence	1120 bp
P24	5'-CGGAAATGTGAATCCCCACC-3'	Downstream sequence in <i>AfuSSUL1</i>	
P25	5'-CGTCCTCTCTTGATGAGTATCG-3'	Upstream sequence in <i>AfuSSUL2</i>	1135 bp
P26	5'-CGAAGAATATCCTCTTGACACCG-3'	pAN7.1 sequence	
P27	5'-GGTTAAGATCCACGTCTTCGGG-3'	pAN7.1 sequence	1063 bp
P28	5'-AAAAGCCCAGAGGATCAGTGC-3'	Downstream sequence in <i>AfuSSUL2</i>	

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 chosen 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 *ssu1*, *ssu11* and *ssu2* 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 *AfuSsu1p*, *AfuSsu11p* and *AfuSsu2p*,

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. YEPD+TA plates containing the desired concentration of sulphite were inoculated with *A. fumigatus* conidia or dermatophyte mycelium. Alternatively, 100 ml liquid YEPD+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 plaques (2 × 10⁴) of a previously constructed *T. rubrum* λEMBL3 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 λEMBL3 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 $\mu\text{mol ml}^{-1}$). Concomitantly, sulphite was detected at concentrations of 0–0.16 $\mu\text{mol ml}^{-1}$ and 0.64–0.80 $\mu\text{mol ml}^{-1}$ 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 $\mu\text{mol ml}^{-1}$, 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 10^6 clones of a previously

Fig. 1. Plasmid construct and predicted outcome of the p Δ Ssu1 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.

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; SceSsu1p, 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.

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 because of differences in intron positions. The three *A. fumigatus* cDNAs were cloned into the *S. cerevisiae* expression vector pYES2 under control of the *GAL* promoter, and the resulting plasmids were transformed into *S. cerevisiae*. Only transformants encoding one of the three transporter genes, corresponding to Afu7g01790, showed increased sulphite resistance on galactose media (Fig. 3). This gene was called *AfuSSU1* (GenBank accession no.

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*

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 (10^6 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 λ EMBL3 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

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.

Fig. 6. Northern blots of *T. rubrum*, *Arth. benhamiae* 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 TruSSUp, AbeSSU1p and AfuSSU1p, respectively, were cloned in pYES2. 28S and 18S rRNAs are indicated to the right of the figure by < and <<, respectively.

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. TruSsulp and AbeSsulp 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.

AfuSsulp, TruSsulp and AbeSsulp are only 20% identical to SceSsulp. In contrast, *A. fumigatus* transporters of the TDT family which are not sulphite efflux pumps are more similar to AfuSsulp (Fig. 2). Alignments of TDT transporters 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

fungus metabolite, sulphite is potentially toxic in the cytoplasm (Thomas & Surdin-Kerjan, 1997). Excess sulphite is generally excreted by fungi after oxidation in the form of inorganic sulphate (Obata & Ishikawa, 1959; Kunert, 1989).

TruSsulp	G	T	F	P	M	G	F	A	T	I	I	N	104–115
AbeSsulp	.	.	.	.	.	.	.	.	.	.	.	.	104–115
AfuSsulp	.	.	.	.	.	.	L	T	.	.	.	.	103–114
SceSsulp	.	.	Y	.	.	.	L	V	.	.	.	.	94–105
AfuSsul1p	A	.	C	.	.	.	.	.	L	.	E	.	102–113
AfuSsul2p	S	.	.	.	.	.	L	.	.	.	V	.	139–150
SpoMaelp	P	.	A	L	L	A	I	.	.	S	.	S	105–116
TruSsulp	L	P	I	G	P	L	G	Q	G	G	F	G	242–253
AbeSsulp	.	.	.	.	.	.	.	.	.	.	.	.	242–253
AfuSsulp	.	.	L	.	.	.	.	.	.	.	Y	.	242–253
SceSsulp	.	L	L	.	.	M	.	.	.	S	.	.	257–268
AfuSsul1p	.	.	L	.	.	.	.	F	.	.	.	.	241–252
AfuSsul2p	.	.	M	.	.	.	.	S	.	T	.	A	278–289
SpoMaelp	I	L	V	S	.	P	S	F	T	.	L	T	242–253

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, AfuSsul1p and AfuSsul2p, and SpoMaelp (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 aetiological 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 β -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.

ACKNOWLEDGEMENTS

We thank Dr Kunlin Zhang for bioinformatic help, and Dr Bertrand Favre, Dr Lee Laurent-Applegate and Dr Massimo Lurati for critical

review of the manuscript and assistance with the English. This work was supported by the Swiss National Foundation for Scientific Research, grant 3100-105313/1.

REFERENCES

- Avram, D. & Bakalinsky, A. T. (1997). *SSU1* encodes a plasma membrane protein with a central role in a network of proteins conferring sulfite tolerance in *Saccharomyces cerevisiae*. *J Bacteriol* **179**, 5971–5974.
- Chambers, S. P., Prior, S. E., Barstow, D. A. & Minton, N. P. (1988). The pMTL *nic⁻* cloning vectors. Improved pUC polylinker regions to facilitate the use of sonicated DNA for nucleotide sequencing. *Gene* **68**, 139–149.
- Cove, D. J. (1966). The induction and repression of nitrate reductase in the fungus *Aspergillus nidulans*. *Biochim Biophys Acta* **113**, 51–56.
- Denikus, N., Orfaniotou, F., Wulf, G., Lehmann, P. F., Monod, M. & Reichard, U. (2005). Fungal antigens expressed during invasive aspergillosis. *Infect Immun* **73**, 4704–4713.
- Donalies, U. E. & Stahl, U. (2002). Increasing sulphite formation in *Saccharomyces cerevisiae* by overexpression of *MET14* and *SSU1*. *Yeast* **19**, 475–484.
- Fumeaux, J., Mock, M., Ninet, B., Jan, I., Bontems, O., Léchenne, B., Lew, D., Panizzon, R. G., Jousson, O. & Monod, M. (2004). First report of *Arthroderma benhamiae* in Switzerland. *Dermatology* **208**, 244–250.
- Grobler, J., Bauer, F., Subden, R. E. & vanVuuren, H. J. J. (1995). The *mae1* gene of *Schizosaccharomyces pombe* encodes a permease for malate and other C4 dicarboxylic acids. *Yeast* **11**, 1485–1491.
- Grossberger, D. (1987). Minipreps of DNA from bacteriophage lambda. *Nucleic Acids Res* **15**, 6737.
- Henderson, J. W., Ricker, R. D., Bidlingmeyer, B. A. & Woodward, C. (2000). *Rapid, Accurate, Sensitive and Reproducible Analysis of Amino Acids*. Agilent technical note no. 5980–1193E.
- Jousson, O., Léchenne, B., Bontems, O., Capoccia, S., Mignon, B., Barblan, J., Quadroni, M. & Monod, M. (2004a). Multiplication of an ancestral gene encoding secreted fungalsin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. *Microbiology* **150**, 301–310.
- Jousson, O., Léchenne, B., Bontems, O., Mignon, B., Reichard, U., Barblan, J., Quadroni, M. & Monod, M. (2004b). Secreted subtilisin gene family in *Trichophyton rubrum*. *Gene* **339**, 79–88.
- Kunert, J. (1972a). Keratin decomposition by dermatophytes: evidence of the sulphitolysis of the protein. *Experientia* **28**, 1025–1026.
- Kunert, J. (1972b). Thiosulphate esters in keratin attacked by dermatophytes in vitro. *Sabouraudia* **10**, 6–13.
- Kunert, J. (1975). Formation of sulphate, sulphite and S-sulphocysteine by the fungus *Microsporum gypseum* during growth on cystine. *Folia Microbiol* **20**, 142–151.
- Kunert, J. (1976). Keratin decomposition by dermatophytes. II. Presence of S-sulphocysteine and cysteic acid in soluble decomposition products. *Z Allg Mikrobiol* **16**, 97–105.
- Kunert, J. (1982). Utilization of L- and DL-cystine by the fungus *Microsporum gypseum*. *Folia Microbiol* **27**, 390–394.
- Kunert, J. (1989). Utilization of L-cystine as a source of carbon and nitrogen by various fungi. *Acta Univ Palacki Olomuc Fac Med* **123**, 351–364.
- Kunert, J. (1992). Effect of reducing agents on proteolytic and keratinolytic activity of enzymes of *Microsporum gypseum*. *Mycoses* **35**, 343–348.

- Kunert, J. (2000). Physiology of keratinophilic fungi. *Rev Iberoam Micol* 17, 77–85.
- Kwong-Chung, K. J. & Bennet, J. E. (1992). *Medical Mycology*. Philadelphia and London: Lea and Febiger.
- Monod, M., Togni, G., Hube, B. & Sanglard, D. (1994). Multiplicity of genes encoding secreted aspartic proteinases in *Candida* species. *Mol Microbiol* 13, 357–368.
- Monod, M., Léchenne, B., Jousson, O., Grand, D., Zaugg, C., Stocklin, R. & Grouzmann, E. (2005). Aminopeptidases and dipeptidyl-peptidases secreted by the dermatophyte *Trichophyton rubrum*. *Microbiology* 151, 145–155.
- Obata, Y. & Ishikawa, Y. (1959). Biochemical studies on sulfur-containing aminoacids. II. Sulfate formation from L-cystine by molds. *J Biochem* 46, 293–295.
- Paris, S. (1994). Isolation of protease negative mutants of *Aspergillus fumigatus* by insertion of a disrupted gene. In *Molecular Biology of Pathogenic Fungi: a Laboratory Manual*, pp. 49–55. Edited by B. Maresca & G. S. Kobayashi. New York: Telos Press.
- Park, H. & Bakalinsky, A. T. (2000). *SSU1* mediates sulphite efflux in *Saccharomyces cerevisiae*. *Yeast* 16, 881–888.
- Park, H., Lopez, N. I. & Bakalinsky, A. T. (1999). Use of sulfite resistance in *Saccharomyces cerevisiae* as a dominant selectable marker. *Curr Genet* 36, 339–344.
- Punt, S. J., Oliver, P. O., Dingemans, M. A., Pouwels, P. H. & van den Hondel, C. A. M. J. J. (1987). Transformation of *Aspergillus* based on hygromycin B resistance marker from *Escherichia coli*. *Gene* 56, 117–124.
- Ruffin, P., Andrieu, S., Biserte, G. & Biguet, J. (1976). Sulphitolysis in keratinolysis. Biochemical proof. *Sabouraudia* 14, 181–184.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Scaringelli, F. P., Saltzman, B. E. & Frey, S. A. (1967). Spectrophotometric determination of atmospheric sulfur dioxide. *Anal Chem* 39, 1709–1726.
- Sherman, F. (1991). Mapping yeast genes. *Methods Enzymol* 194, 38–57.
- Thomas, D. & Surdin-Kerjan, Y. (1997). Metabolism of sulfur amino acids in *Saccharomyces cerevisiae*. *Microbiol Mol Biol Rev* 61, 503–532.
- Tilburn, J., Scazzocchio, C., Taylor, G. G., Zabicky-Zissman, J. H., Lockington, R. A. & Davies, R. W. (1983). Transformation by integration in *Aspergillus nidulans*. *Gene* 26, 205–221.
- Viklund, H. & Elofsson, A. (2004). Best alpha-helical transmembrane protein topology predictions are achieved using hidden Markov models and evolutionary information. *Protein Sci* 13, 1908–1917.
- Walter, E. G., Weiner, J. H. & Taylor, D. E. (1991). Nucleotide sequence and overexpression of the tellurite-resistance determinant from the IncHII plasmid pHH1508a. *Gene* 101, 1–7.
- Weitzman, I. & Summerbell, R. C. (1995). The dermatophytes. *Clin Microbiol Rev* 8, 240–259.

Edited by: N. L. Glass