Closely related dermatophyte species produce different patterns of secreted proteins

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Abstract

Dermatophytes are the most common infectious agents responsible for superficial mycosis in humans and animals. Various species in this group of fungi show overlapping characteristics. We investigated the possibility that closely related dermatophyte species with different behaviours secrete distinct proteins when grown in the same culture medium. Protein patterns from culture filtrates of several strains of the same species were very similar. In contrast, secreted protein profiles from various species were different, and so a specific signature could be associated with each of the six analysed species. In particular, protein patterns were useful to distinguish *Trichophyton tonsurans* from *Trichophyton equinum*, which cannot be differentiated by ribosomal DNA sequencing. The secreted proteases Sub2, Sub6 and Sub7 of the subtilisin family, as well as Mep3 and Mep4 of the fungalisin family were identified. *SUB6*, *SUB7*, *MEP3* and *MEP4* genes were cloned and sequenced. Although the protein sequence of each protease was highly conserved across species, their level of secretion by the various species was not equivalent. These results suggest that a switch of habitat could be related to a differential expression of genes encoding homologous secreted proteins.

Introduction

Dermatophytes are a group of fungi responsible for parasitic infections of the keratinized tissues (skin, hair and nails) in humans and animals. Dermatophytoses, commonly named clinically ringworm or tinea, are due to the ability of these fungi to obtain nutrients from keratinized material. On the basis of their primary habitat, dermatophytes are classified as anthropophilic (humans), zoophilic (animals) or geophilic (soil) (Weitzman & Summerbell, 1995; Weitzman & Padhye, 1996). However, these phylogenetically and taxonomically closely related fungi often show overlapping characteristics. Some zoophilic and geophilic dermatophyte species like *Trichophyton verrucosum* and *Microsporum gypseum* can infect humans (Lateur, 2000; Khosravi & Mahmoudi, 2003). The zoophilic species *Trichophyton equinum* and the anthropophilic species *Trichophyton tonsurans* possess almost identical internal transcribed spacer (ITS) regions and ribosomal DNA sequences (Gräser *et al.*, 1999; Summerbell *et al.*, 1999). In addition, isolates of *Trichophyton rubrum* and of the anthropophilic variety of *Arthroderma vanbreuseghemii* (*Trichophyton interdigitale*) (Gräser *et al.*, 1999) sometimes show the same morphology when grown on Sabouraud's medium, thus leading to their misidentification (Kane & Fischer, 1971; Mahmoud *et al.*, 1996). Moreover, the inflammatory reactions produced by dermatophytes in the host have been shown to vary from mild to severe according to the primary habitat of the fungus, the anatomic location of the infection and the host's reactions to the metabolites secreted by the fungus (Wagner & Sohnle, 1995; Nissen *et al.*, 1998; Mari *et al.*, 2003).

A fundamental problem concerning dermatophytes is to obtain a comprehensive view of the attributes conferring specialization, that is, virulence as well as the ability to survive and thrive in a new host population. It is likely that patterns of secreted proteins are closely related to selective functions and ecological background in dermatophytes. The identification and characterization of novel secreted dermatophyte proteases could allow a better understanding of the interactions existing between dermatophytes and their environment.

In this study, we have investigated the ability of dermatophytes with different habitats to produce distinct patterns of proteins in culture filtrates. We have analysed the proteins secreted by six closely related species: *A. vanbreuseghemii* and five *Trichophyton* species. The six species were grown in

a medium that promotes secretion of proteases. The examination of protein profiles, the identification of some orthologous secreted proteases and the comparison of their amino acid sequences indicated that each species secretes a specific panel of homologous proteins. Therefore, the secreted patterns were also a useful tool to differentiate closely related species.

Materials and methods

Species and strains

The species and strains of dermatophytes used in this study are described in Table 1. All strains, except those of *T. equinum*, were isolated from patients at the University Hospital of Lausanne (Switzerland). *Trichophyton equinum* strains were isolated from horse ringworm and skin dermatitis at the Veterinary Faculty of Liège (Belgium, Liège; isol. det. B. Mignon, 1998) and at the Centre of Diseases Detection in cattle (Belgium, Hainaut; isol. det. P.E. Lagneau, 2003). All strains were given preliminary or, where possible, definitive identification on the basis of the macroscopic appearance and the microscopic characteristics of the cultures. Species determination was usually confirmed by sequencing of the 28S rRNA gene (Ninet *et al.*, 2003; Table 1). The previously called *Trichophyton mentagrophytes* Type III by Ninet *et al.* (2003) are named *A. vanbreuseghemii* (zoophilic strains) in this study.

A microsatellite marker (T1) was used for the typing of *T. rubrum* (Ohst *et al.*, 2004). A type B polymorphism was shown by all strains (Table 1).

Growth media

All isolates were initially grown on Sabouraud agar (Bio-Rad). To promote the production of proteases, a plug of freshly growing mycelium from Sabouraud agar was poured into 100 mL of either 0.2% soy protein (SP) (Supro 1771, Protein Technologies International), or 0.2% keratin (KP) (Merck 5201) dissolved in water and sterilized for 15 min at 120 °C (Jousson *et al.*, 2004a). Keratin-soy liquid medium (KSP) was prepared by adding 10 mL of SP medium to 90 mL of KP medium (Jousson *et al.*, 2004a). Cultures were usually incubated for 16 days at 30 °C without shaking. The proteolytic activity was measured using resorufin-labelled casein (Roche Diagnostics) (Jousson *et al.*, 2004a).

PCR and DNA sequencing

To amplify the genes *SUB6* and *SUB7*, encoding secreted serine proteases of the subtilisin family, as well as *MEP3* and *MEP4*, encoding secreted metalloproteases of the fungalisin family from various dermatophytes, homologous primers derived from the corresponding genomic DNA sequences of *T. rubrum* were used: 5′-ATGGGTTTCATCACCAAAGCCATT and 5′-ATTTGCCGCTGCCGTTGTAGATAA for *SUB6*; 5′-ATGGGTTTCATCACCAAGGCC and 5′-CATGCCGGATCCGTTGTTGATGAG for *SUB7*; 5′-TCGCCACAACAGC

Table 1. Dermatophyte strains used for the analysis of secreted protein profiles

Strain*	Ident. according to 28S sequence	Source	Country	Gene	GenBank† Accession n°
LAU931	*A. vanbreuseghemii*‡	*Tinea capitis*	Swiss	*SUB6; SUB7*	AJ430840; DQ382271
LAU2434	*A. vanbreuseghemii*‡	*Tinea faciei*	Swiss	*MEP3*	AY283574;
LAU2642	*A. vanbreuseghemii*‡	*Tinea corporis*	Swiss	*MEP4*	AY283576
IHEM15219	*T. equinum*	Horse ringworm	Belgium	*SUB6*; *SUB7*	DQ382269; DQ382272
IHEM20668	*T. equinum*	Horse skin	Belgium	*MEP3*	DQ409176
IHEM20669	*T. equinum*	Horse skin	Belgium	*MEP4*	DQ384953
LAU704	*T. tonsurans*	*Tinea capitis*	Swiss	*SUB6*; *SUB7*	AY910749; DQ382273
LAU1405	*T. tonsurans*	*Tinea capitis*	Swiss	*MEP3*	DQ384950
ER6906	*T. tonsurans*	*Tinea capitis*	England	*MEP4*	DQ384954
LAU1485	*T. rubrum*§	*Tinea unguium*	Swiss	*SUB6*; *SUB7*	AF420485; AF407184
LAU1738	*T. rubrum*§	*Tinea unguium*	Swiss	*MEP3*	AY283569
LAU1745	T. rubrum§	*Tinea pedis*	Swiss	*MEP4*	AF407191
LAU228	*T. soudanense*	*Tinea capitis*	Swiss	*SUB6*; *SUB7*	DQ382270; DQ382274
LAU556	*T. soudanense*	*Tinea capitis*	Swiss	*MEP3*	DQ384951
LAU2221	*T. soudanense*	*Tinea capitis*	Swiss	*MEP4*	DQ384955
LAU209	*T. violaceum*	*Tinea capitis*	Swiss	*SUB7*	DQ382275
LAU819	*T. violaceum*	*Tinea capitis*	Swiss	*MEP3*	DQ384952
LAU551	*T. violaceum*	*Tinea capitis*	Swiss	*MEP4*	DQ384956

*All strains are available at the CBS public collection.

†Sequences are related to the corresponding species but not to a particular strain.

‡*Arthroderma vanbreuseghemii* correspond to *T. mentagrophytes* type III (AF506034) according to Ninet *et al.* (2003).

§All *T. rubrum* strains showed a type B polymorphism according to Ohst *et al.* (2004).

GAGACTG and 5′-TTAGCAGCCTGGTGGGAG for *MEP3*; and 5′-GATGCAAACAAGAACGACAAA and 5′-AGCAGC CAGCAGGAAGCTCGT for *MEP4*. Primers were made by Microsynth (Balgach, Switzerland). PCRs were performed in a total volume reaction of 50 μL containing 0.1 μg of target genomic DNA with 1.5 U of Ampli*Taq* DNA polymerase (Roche). Amplified DNA fragments were cloned into the pDrive Cloning Vector (Qiagen). Plasmids were sequenced by Synergene Biotech GmbH (Zurich, Switzerland) and FASTERIS SA (Geneva, Switzerland).

Protein extract analysis by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and Western blot

The mycelium was separated from culture medium by paper filtration. Secreted proteins in 10 mL filtrate were precipitated on ice for 10 min with 10% trichloroacetic acid. After centrifugation, the protein pellet was dissolved in 20 μL of 20 mM Tris-HCl, pH 7.4. The pH was adjusted to 8.0 with NaOH 1N. Concentrated extracts were analysed by SDS-PAGE on 4–12% polyacrylamide gradient gels (NuPAGE Novex Precast Gels, Invitrogen). Gels were stained with Coomassie brilliant blue R-250 (Bio-Rad).

Immunoblots were performed using rabbit antisera and peroxidase-conjugated goat antirabbit IgG (Amersham Pharmacia) as secondary labelled antibody. This conjugated antibody was detected by chemoluminescence with the ECL system (Amersham Biosciences).

Polyclonal antibodies

Anti-Mep serum recognizing Mep3 and Mep4 was previously described (Doumas *et al.*, 1998). To raise polyclonal antibodies large polypeptides corresponding to sequences from *T. rubrum* Sub2 (res. 201 to 424, GenBank no. AY343500), Sub6 (res. 265 to 412, GenBank no. AF420485) and subtilisin 7 (Sub7) (res. 192 to 344, GenBank no. AF407184) were produced in *Escherichia coli* BL21 using the pET expression system from Novagen (Darmstadt, Germany). Heterologous His_6-tagged peptides were extracted with guanidine hydrochloride buffer and Ni-NTA resin columns (Qiagen, Hilden, Germany) (Reichard *et al.*, 2006). Rabbits were immunized with these purified polypeptides at Eurogentec (Liege, Belgium).

Protein identification by LC-MS/MS

Stained bands were excised and in-gel digested with trypsin (Promega) on an Investigator ProGest robotic workstation (Perkin Elmer Life Sciences) as described (Wilm *et al.*, 1996). Concentrated digests were analysed by reversed-phase LC-MS/MS on an SCIEX QSTAR Pulsar (Concord, Ontario) mass spectrometer interfaced to an LC-Packings Ultimate nanoHPLC system (Amsterdam, the Netherlands) (Owen *et al.*, 2005). Alternatively, digests were dried and resuspended in an alpha-cyano-hydroxycinnamic acid matrix for MALDI-MS-MS analysis on a 4700 Proteomics Analyser (Applied Biosystems, Framingham, MA) set to perform MS/MS analysis on the 20 most intense signals. Peptide fragmentation spectra were used for searching the fungi subset of the Uniprot 7.5 (www.expasy.org) database using Mascot (http://www.matrixscience.com). In one case (*T. tonsurans* Mr 37 000 band), for confirmation, a subset of MS/MS spectra were manually interpreted to derive a set of sequence tags and then used for homology searches in the NCBI nr database with MS-BLAST (http://www.dove.embl-heidelberg.de/Blast2/msblast.html) (Shevchenko *et al.*, 2001).

Results

Selection of the best medium allowing dermatophytes to grow and secrete proteases

In order to induce a high proteolytic activity in dermatophytes, three different protein liquid media were tested: SP, KP and KSP media. We compared the growth of *A. vanbreuseghemii, T. rubrum* and *Trichophyton soudanense* species in these media, as well as proteolytic activity present in the culture filtrates. Dermatophytes grew the fastest in SP medium, with maximal proteolytic activities measured at 16 ± 2 days of culture. KP medium could not sustain fungal growth. Supplementation of KP medium with 0.02% of SP, KSP medium, could circumvent this problem. Nevertheless, growth in KSP medium was slow and maximal proteolytic activity was not observed before 5 weeks of incubation. SP medium was therefore used in all subsequent experiments.

Comparison of secreted protein profiles produced by dermatophytes

Culture supernatants were collected after 16 days of growth and secreted proteins concentrated and size-fractionated on SDS-PAGE, which was stained with Coomassie blue (see Materials and methods). Although ten strains per species were analysed, the results obtained with three of them are shown as representative examples. Protein profiles secreted by strains of the same species were similar (Fig. 1). Only minor differences were observed concerning the intensity of one or two proteins having the same electrophoretic mobility. As an example, see the band at Mr 16 000 (not identified yet) in *T. rubrum* strains 1485, 1738 and 1745 (Fig. 1). In contrast, different species produced distinct secreted protein patterns (Fig. 1). In particular, profiles exhibited by the closely related *T. equinum* and *T. tonsurans* were remarkably different. *Trichophyton equinum* secreted a large spectrum of proteins having both diverse sizes and intensities, whereas a

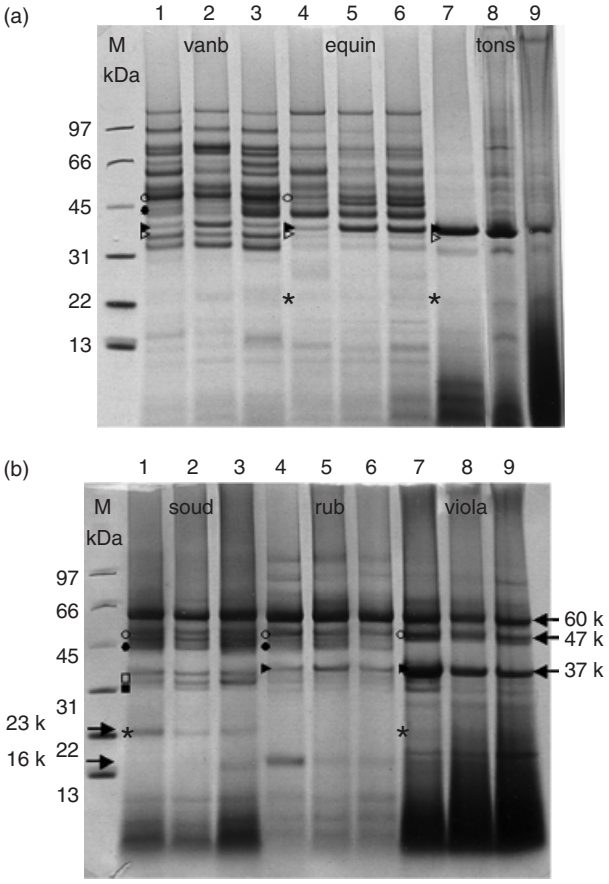

Fig. 1. Protein profiles secreted by *Arthroderma vanbreuseghemii* (vanb), *Trichophyton equinum* (equin), *Trichophyton tonsurans* (tons), *Trichophyton soudanense* (soud), *Trichophyton rubrum* (rub) and *Trichophyton violaceum* (viola). Three strains per species are illustrated. (a): (1) vanb 931, (2) vanb 2434, (3) vanb 2642; (4) equin 15219 (5) equin 20668 (6) equin 20669; (7) tons 704, (8) tons 1405, (9) tons 6906. b: (1) soud 228, (2) soud 556, (3) soud 2221; (4) rub 1485, (5) rub 1738, (6) rub 1745; (7) viola 551, (8) viola 209, (9) viola 819. Standard molecular mass marker is indicated in lane M. The equivalent of 2 mL of culture supernatant 500-fold concentrated (4 μL) was loaded in a total volume of 16 μL. The 4–12% gradient polyacrylamide SDS-PAGE was stained with Coomassie blue. Bands marked by • and ○ correspond to Mep3 and Mep3 and/or Mep4-glycosylated proteins, respectively. Bands marked by: ▲, △ and * correspond to Sub7, Sub2 and Sub6 proteins, respectively. The five proteins have been confirmed by Western blotting (see Fig. 2). In lane 1A, we also show the localization of Sub3 (□) and Sub4 (■) proteins (Jousson *et al*., 2004b).

single major protein of Mr 37 000 was secreted by *T. tonsurans*. *Arthroderma vanbreuseghemii* showed a protein secretion profile close to *T. equinum*. Differences between *A. vanbreuseghemii* and *T. equinum* profiles were not in the number of detected proteins but rather in band intensities.

The closely related *T. rubrum*, *T. soudanense* and *Trichophyton violaceum* showed secreted protein profiles with some analogies. Nevertheless, a closer examination revealed some distinct features for each species. Profiles of *T. violaceum* were characterized by a high secretion level of mainly three proteins of Mr 60 000, 47 000 and 37 000. Profiles of *T. rubrum* contained these same three bands but they were less intense, while *T. soudanense* profiles were characterized by the absence of the Mr 37 000 protein (Figs 1 and 2a). Moreover, *T. soudanense* secreted an Mr 23 000 protein, which was either absent or very faint in profiles from other species (Figs 1 and 2c).

Identification of some homologous proteases

We tried to identify the major protein (Mr 37 000) secreted by *T. tonsurans* and the proteins having the same electrophoretic mobility in *T. rubrum, T. equinum* and *T. violaceum* (Fig. 1) using LC-MS/MS. The obtained spectra were deciphered to derive sequence tags of 5–10 amino acids in length for each protein (see Table 2). Homology searches in different protein databases indicated that independently of the species, all obtained sequence tags were similar or identical to the *T. rubrum* protein, Sub7 (Jousson *et al.*, 2004b). To confirm these conclusions, we performed Western blot analyses of the six species secretomes (Fig. 2a). Anti-Sub7 serum indeed detected an Mr 37 000 protein in the culture medium from all species except for *T. soudanense* (Fig. 2a). Because Sub7 is closely related to subtilisin 2 (Sub2) and subtilisin 6 (Sub6) proteins (Jousson *et al.*, 2004b), we also analysed secretomes with anti-Sub2 and anti-Sub6 sera. Sub2 protein (Mr 36 000) was unambiguously detected only in the culture media from the closely related species *A. vanbreuseghemii*, *T. equinum* and *T. tonsurans* (Fig. 2b). The identity of the extra upper immunogenic band in *A. vanbreuseghemii* lane is unknown. Anti-Sub6 serum detected two major bands of Mr 40 000 and Mr 23 000 in the secretome of *T. soudanense* (Fig. 2c). The *T. rubrum* Sub6 protein has a theoretical molecular mass of 42 709 Da and 40 710 Da without the signal peptide. Our results suggest that the secreted Sub6 (Mr 40 000) is proteolysed to a smaller form of Mr 23 000. Anti-Sub6 serum appears to cross-react weakly with Sub7 (Mr 37 000) (Fig. 2a and c).

Previously, we showed the secretion of the metalloprotease 3 (Mep3) and metalloprotease 4 (Mep4) by *T. rubrum* and *A. Vanbreuseghemii* (named *T. mentagrophytes*) (Jousson *et al.*, 2004a). Here, we indeed identified by LC-MS/MS the secreted band of Mr 43 000 in *T. rubrum* profile as Mep3. We used the anti-Mep serum to further analyse the secretion of Mep3 and Mep4 proteases by other *Trichophyton* species (Jousson *et al.*, 2004a). Anti-Mep serum detected Mep3 (Mr 43 000) only in the culture medium from *T. rubrum*, *A. vanbreuseghemii* and *T. soudanense* (Fig. 2d). All tested species secreted immunoreactive band(s) with Mr 47 000 in their culture medium, although *T. tonsurans* to a lower level than the other species (Fig. 2d). This band(s) could be

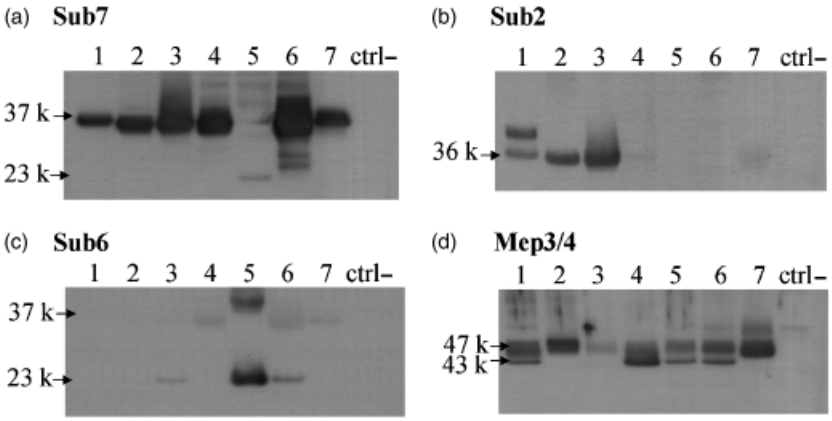

Fig. 2. Western blot analysis on culture supernatants from dermatophyte species. Proteins from the culture supernatant of dermatophyte species were concentrated 500-fold, and 4 μL of this solution was loaded on a 4–12% gradient SDS-PAGE. (a) Sub7: (1) vanb 931, (2) equin 20668, (3) tons 704, (4) rub 1738, (5) soud 228, (6) viola 209, (7) benh 2037.(b) Su2: (1) vanb 931, (2) equin 20668, (3) tons 704, (4) rub 250, (5) rub 1738, (6) soud 228, (7) viola 209. (c) Sub6: (1) vanb 931, (2) equin 20668, (3) tons 704, (4) rub 1738, (5) soud 228, (6) viola 209, (7) benh 2037. (d) Mep3/4: (1) vanb 931, (2) equin 20668, (3) tons 704, (4) rub 250, (5) rub 1738, (6) soud 228, (7) viola 209. As control, 1.0 μg of purified Sub4 protein was loaded in parallel (lane 8). Proteins were detected using antisera raised against portions of the respective heterologous secreted Sub 7, 6 and 2, as well as against portion of Mep3/4 proteins (see Material and methods). An additional dermatophyte species, *Arthroderma benhamiae*, was tested for Sub7 and Sub6. A slight cross-reaction was observed between antibodies detecting Sub7 and Sub6 proteins (Fig. 2a and c).

Table 2. Identification of secreted proteins by MS

Species	Protein Acc. n°	Protein description	Mascot protein score	Calc. MW	eq. Cov.	calc. mr	Mass error	Pep Score	Peptide sequence
T. tonsurans	Q5VJ71_9EURO	Protease SUB7	242	41 783	14.51	105.72	0.19	56.64	TVAASTEQDGK
						1379.32	0.68	54.1	ICTVAASTEQDGK
						1637.67	0.74	37.25	ATPHVAGLGAYLIGLGK
						1683.14	0.3	80.05	AIEQQDNVPSWGLAR
						1683.35	0.51	40.24	AIEQQDNVPSWGLAR
						2812.19	0.73	23.15	PGGGSQVLSGTSMATPHVAGLG AYLIGLGK
	Q8NID9_TRIRU	Protease SUB7	136	41 808	11.2	1637.67	0.74	37.25	ATPHVAGLGAYLIGLGK
						1683.14	0.3	80.05	AIEQQDNVPSWGLAR
						1683.35	0.51	40.24	AIEQQDNVPSWGLAR
T. rubrum	Q5VJ71_9EURO	Protease SUB7	476	41 783	24	1073.49	−0.03	50.9	GGGPGLCDTIK
						1230.72	−0.02	64.18	VAGLGAYLIGLGK
						1682.83	−0.01	86.19	AIEQQDNVPSWGAR
						1775.87	0	87.06	QMAIDVIQNPGASTTSK
						1844.85	0	60.95	VIDTGTDIQHEEFEGR
						2251.12	0.07	131.76	AVANMSLGGAFSQASNDAAAAIAQ
T. equinum	Q5VJ71_9EURO	Protease SUB7	52	41 783	3.8	1682.87	0.03	52.47	AIEQQDNVPSWGLAR
T.violaceum	Q8NID9_TRIRU	Protease SUB7	315	41 808	13	1073.5	−0.02	68.07	GGGPGLCDTIK
						1759.91	0.04	60.27	QMAIDVIQNPGASTTSK
						2235.18	0.08	186.62	AVANMSLGGAFSQASNDAAAAIAK

explained by the presence of Mep4 and/or Mep3 and Mep4 glycosylated forms as described (Jousson *et al.*, 2004a).

Highly conserved amino acid sequences of orthologous secreted proteases

The proteases Sub7, Mep3 and Mep4 were shown to be secreted by most of the six analysed species (Fig. 2a and d). The protease Sub6 was predominantly secreted by *T. soudanense*. To determine their protein sequences, all four genes were amplified, more precisely the fragment corresponding to the mature domain of Mep3 and Mep4, along with the complete coding sequence of Sub6 and Sub7. The *SUB7*, *MEP3* and *MEP4* genes were sequenced from all the six species. *SUB6* gene was not successfully amplified from *T. violaceum* genomic DNA. Alignment of their amino acid

Table 3. Polymorphism among the protein sequences of Sub6, Sub7, Mep3 and Mep4

	Sub6		Sub7		Mep3		Mep4	
Species	Homology between closely species	Total homology among the six species	Homology between closely species	Total homology among the six species	Homology between closely species	Total homology among the six species	Homology between closely species	Total homology among the six species
A. vanbreuseghemii 2434 *T. equinum* 15219 *T. tonsurans* 704	100% similarity 97.1% identity	99.8% similarity 90.3% identity	100% similarity 99.0% identity	99.8% similarity 91.5% identity	100% similarity 100% identity	100% similarity 96.8% identity	100% similarity 99.0% identity	100% similarity 94.6% identity
T. rubrum 17938 *T. soudanense* 228 *T. violaceum* 209	100% similarity 100% identity		100% similarity 99.3% identity		100% similarity 100% identity		100% similarity 99.5% identity	

sequences revealed a strong similarity, sometimes even total identity, across species. Nevertheless, two groups of sequences could be distinguished (Table 3). The first comprises *A. vanbreuseghemii*, *T. equinum* and *T. tonsurans* and the second *T. rubrum*, *T. soudanense* and *T. violaceum*. Interestingly, a similar distinction could be deduced from the secretome profiles (Fig. 1).

Discussion

Dermatophytes grow exclusively in the *stratum corneum* and use keratin and different cross-linked proteins of the cornified cell envelope as substrates. It is thus reasonable to postulate that during infection, dermatophytes secrete a complete battery of endo- and exo-proteases to degrade keratinized structures into short peptides and free amino acids to be used as nutrients by the fungus. Here, we have shown that closely related dermatophyte species secrete different protein patterns when grown in the same culture medium. The analysis of the secreted protein profiles allowed the distinction of closely related species with different ecology such as *T. tonsurans* and *T. equinum* or *T. rubrum*, *T. soudanense* and *T violaceum*.

In recent years, sophisticated advances in molecular biology have provided essential tools to mycologists to explore and understand the relationship among dermatophytes. The phylogenetic delineation of dermatophytes has been improved using genetic techniques such as rRNA gene PCR-restriction fragment length polymorphism analysis (Mochizuki *et al.*, 2003a, b; Machouart *et al.*, 2006), DNA sequence analysis of the 28S RNA gene and the ITS regions (Summerbell *et al.*, 1999; Gupta *et al.*, 2001; Ninet *et al.*, 2003). However, the results of these techniques led some investigators to recommend that a number of dermatophyte species were reduced to synonymy with other well-established species, thus creating confusion. One of the most interesting examples of controversy is the disagreement existing in the classification of *T. equinum* and *T. tonsurans*, which, based on results of the sequence analysis of the highly variable ITS regions, were described as synonymous (De Hoog *et al.*, 1998; Gräser *et al.*, 1999). Differences in morphology, physiology, ecology and types of human infections caused by these fungi in fact support a clear separation between *T. equinum* and *T. tonsurans* (Woodgyer, 2004). In our study, we have demonstrated by comparing the secreted protein patterns of *T. equinum* and *T. tonsurans* that both fungi are doubtlessly two different species.

For the first time, the proteases Sub2 and Sub6 were shown to be secreted by dermatophytes. We also found that most *Trichophyton* species and *A. vanbreuseghemii*, in addition to *Arthroderma benhamiae* (Jousson *et al.*, 2004b), secrete Sub7. In the same way, Mep3 and Mep4 were immunologically detected in the culture filtrates of the analysed dermatophyte species. These biochemical results are corroborated by the demonstration of the presence of *SUB6*, *SUB7*, *MEP3* and *MEP4* genes in the genome of all *Trichophyton* and the *Arthroderma* species investigated, except for SUB6 gene in *T. violaceum*. Orthologous secreted proteases were differently expressed by dermatophytes, even though they share a very high degree of similarity and identity (this study and Ref. Jousson *et al.*, 2004a, b). All these results suggest that ecological switching could be related to a differential expression of genes encoding secreted proteins, particularly, proteases, rather than genetic divergences of the genes encoding orthologous proteins.

Differential secretion of proteins by dermatophyte species could also be responsible for the variable inflammation caused by the infectious agent within the host. As a general rule, zoophilic species (like *T. equinum*) cause lesions that are more inflammatory than those caused by anthropophilic species (like *T. tonsurans*), but are also more likely to resolve spontaneously (Takatori & Ichijo, 1985; Brasch *et al.*, 1998). The analyses of the genes, the amino acid sequences and the secreted protein patterns of *T. equinum* and *T. tonsurans* support the idea that different inflammatory responses could be due to different levels of antigen secretion and not due to the amino acid differences between orthologous antigens (Fig. 1 and Table 3). Therefore, further identification of secreted proteases by dermatophytes is warranted.

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References

Brasch J, Folster-Holst R & Christophers E (1998) Tinea caused by *Trichophyton equinum*. *Hautarzt* **49**: 397–402.

De Hoog GS, Bowman B, Gräser Y, Haase G, El Fari M, Gerrits van den Ende AH, Melzer-Krick B & Untereiner WA (1998) Molecular phylogeny and taxonomy of medically important fungi. *Med Mycol* **36**: (Suppl 1): 52–56.

Doumas A, van den Broek P, Affolter M & Monod M (1998) Characterization of the prolyl dipeptidyl peptidase gene (dppIV) from the koji mold *Aspergillus oryzae*. *Appl Environ Microbiol* **64**: 4809–4815.

Gräser Y, Kuijpers AF, Presber W & De Hoog GS (1999) Molecular taxonomy of *Trichophyton mentagrophytes* and *T. tonsurans*. *Med Mycol* **37**: 315–330.

Gupta AK, Kohli Y & Summerbell RC (2001) Variation in restriction fragment length polymorphisms among serial isolates from patients with *Trichophyton rubrum* infection. *J Clin Microbiol* **39**: 3260–3266.

Jousson O, Lechenne B, Bontems O, Capoccia S, Mignon B, Barblan J, Quadroni M & Monod M (2004a) Multiplication of an ancestral gene encoding secreted fungalysin preceded species differentiation in the dermatophytes *Trichophyton* and *Microsporum*. *Microbiology* **150**: 301–310.

Jousson O, Lechenne 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.

Kane J & Fischer JB (1971) The differentiation of *Trichophyton rubrum* and *T. mentagrophytes* by use of Christensen's urea broth. *Can J Microbiol* **17**: 911–913.

Khosravi AR & Mahmoudi M (2003) Dermatophytes isolated from domestic animals in Iran. *Mycoses* **46**: 222–225.

Lateur N (2000) Dermatophytoses due to domestic animals. *Rev Med Brux* **21**: A237–A241.

Machouart M, Larche J, Burton K *et al.* (2006) Genetic identification of the main opportunistic mucorales by PCR-restriction fragment length polymorphism. *J Clin Microbiol* **44**: 805–810.

Mahmoud AL, El-Shanawany AA & Omar SA (1996) Factors affecting growth and urease production by *Trichophyton* spp. *Mycopathologia* **135**: 109–113.

Mari A, Schneider P, Wally V, Breitenbach M & Simon-Nobbe B (2003) Sensitization to fungi: epidemiology, comparative skin tests, and IgE reactivity of fungal extracts. *Clin Exp Allergy* **33**: 1429–1438.

Mochizuki T, Ishizaki H, Barton RC, Moore MK, Jackson CJ, Kelly SL & Evans EG (2003a) Restriction fragment length polymorphism analysis of ribosomal DNA intergenic regions is useful for differentiating strains of *Trichophyton mentagrophytes*. *J Clin Microbiol* **41**: 4583–4588.

Mochizuki T, Tanabe H, Kawasaki M, Ishizaki H & Jackson CJ (2003b) Rapid identification of *Trichophyton tonsurans* by PCR-RFLP analysis of ribosomal DNA regions. *J Dermatol Sci* **32**: 25–32.

Ninet B, Jan I, Bontems O, Lechenne B, Jousson O, Panizzon R, Lew D & Monod M (2003) Identification of dermatophyte species by 28S ribosomal DNA sequencing with a commercial kit. *J Clin Microbiol* **41**: 826–830.

Nissen D, Petersen LJ, Esch R, Svejgaard E, Skov PS, Poulsen LK & Nolte H (1998) IgE-sensitization to cellular and culture filtrates of fungal extracts in patients with atopic dermatitis. *Ann Allergy Asthma Immunol* **81**: 247–255.

Ohst T, de Hoog S, Presber W, Stavrakieva V & Gräser Y (2004) Origins of microsatellite diversity in the *Trichophyton rubrum–T. violaceum* clade (Dermatophytes). *J Clin Microbiol* **42**: 4444–4448.

Owen HR, Quadroni M, Bienvenut W, Buerki C & Hottiger MO (2005) Identification of novel and cell type enriched cofactors of the transcription activation domain of RelA (p65 NF-kappaB). *J Proteome Res* **4**: 1381–1390.

Reichard U, Lechenne B, Asif AR, Streit F, Grouzmann E, Jousson O & Monod M (2006) Sedolisins, a new class of secreted proteases from *Aspergillus fumigatus* with endoprotease or tripeptidyl-peptidase activity at acidic pHs. *Appl Environ Microbiol* **72**: 1739–1748.

Shevchenko A, Sunyaev S, Loboda A, Bork P, Ens W & Standing KG (2001) Charting the proteomes of organisms with unsequenced genomes by MALDI-quadrupole time-of-flight mass spectrometry and BLAST homology searching. *Anal Chem* **73**: 1917–1926.

Summerbell RC, Haugland RA, Li A & Gupta AK (1999) rRNA gene internal transcribed spacer 1 and 2 sequences of asexual, anthropophilic dermatophytes related to *Trichophyton rubrum*. *J Clin Microbiol* **37**: 4005–4011.

Takatori K & Ichijo S (1985) Human dermatophytosis caused by *Trichophyton equinum*. *Mycopathologia* **90**: 15–19.

Wagner DK & Sohnle PG (1995) Cutaneous defenses against dermatophytes and yeasts. *Clin Microbiol Rev* **8**: 317–335.

Weitzman I & Summerbell RC (1995) The dermatophytes. *Clin Microbiol Rev* **8**: 240–259.

Weitzman I & Padhye AA (1996) Dermatophytes: gross and microscopic. *Dermatol Clin* **14**: 9–22.

Wilm M, Shevchenko A, Houthaeve T, Breit S, Schweigerer L, Fotsis T & Mann M (1996) Femtomole sequencing of proteins from polyacrylamide gels by nano-electrospray mass spectrometry. *Nature* **379**: 466–469.

Woodgyer A (2004) The curious adventures of *Trichophyton equinum* in the realm of molecular biology: a modern fairy tale. *Med Mycol* **42**: 397–403.