MfLIP1, a gene encoding an extracellular lipase of the lipid-dependent fungus *Malassezia furfur*

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Malassezia furfur is a dimorphic fungus and a member of the normal cutaneous microflora of humans. However, it is also a facultative pathogen, associated with a wide range of skin diseases. One unusual feature of *M. furfur* is an absolute dependency on externally provided lipids which the fungus hydrolyses by lipolytic activity to release fatty acids necessary for both growth and pathogenicity. In this study, the cloning and characterization of the first gene encoding a secreted lipase of *M. furfur* possibly associated with this activity are reported. The gene, *MfLIP1*, shows high sequence similarity to other known extracellular lipases, but is not a member of a lipase gene family in *M. furfur*. *MfLIP1* consists of 1464 bp, encoding a protein with a molecular mass of 54.3 kDa, a conserved lipase motif and an N-terminal signal peptide of 26 aa. By using a genomic library, two other genes were identified flanking *MfLIP1*, one of them encoding a putative secreted catalase, the other a putative amine oxidase. The cDNA of *MfLIP1* was expressed in *Pichia pastoris* and the biochemical properties of the recombinant lipase were analysed. MfLip1 is most active at 40 °C and the pH optimum was found to be 5–8. The lipase hydrolysed lipids, such as Tweens, frequently used as the source of fatty acids in *M. furfur* media, and had minor esterase activity. Furthermore, the lipase is inhibited by different bivalent metal ions. This is the first molecular description of a secreted lipase from *M. furfur*.

INTRODUCTION

The dimorphic fungus *Malassezia furfur* is a ubiquitous colonizer of the human skin. Although considered to be a harmless commensal under normal circumstances, it is also an opportunistic pathogen. As such, it has been associated with various diseases ranging from the pigmentation disorder pityriasis versicolor to atopic dermatitis and catheter-associated sepsis (Crespo Erchiga & Delgado Florencio, 2002; Gueho *et al.*, 1998; Gupta *et al.*, 2004).

With the notable exception of *Malassezia pachydermatis*, all known *Malassezia* species require externally provided lipids for growth. It has been shown that this lipid dependency is due to a defect in the synthesis of myristic acid, which serves as the precursor of long-chain fatty acids (Porro *et al.*, 1976; Shifrine & Marr, 1963). Even so, the outermost layer of the complex cell wall consists mainly of lipids (Mittag, 1995), which are thought to be involved in the pathogenesis of this fungus. Similar to the polysaccharide capsule of *Cryptococcus neoformans* (Ellerbroek *et al.*, 2004), this lipid layer seems to protect *M. furfur* from phagocytosis (Ashbee &

Evans, 2002) and downregulates the inflammatory immune response (Kesavan *et al.*, 2000). In addition, adhesion to host cells may be mediated by the hydrophobicity of the lipid-rich cell wall (Mittag, 1995) as has been shown for some aerobic coryneform bacteria (Bojar *et al.*, 2004). Thus, the ability to metabolize lipids and to integrate the fatty acids into the fungal cell wall is essential for growth and survival in a host environment and contributes notably to the pathogenicity of *M. furfur*. Therefore, the enzymes necessary for these activities can be considered as virulence factors.

Lipases (EC 3.1.1.3) catalyse the hydrolysis of the ester bonds of triacylglycerols, thereby releasing free fatty acids. Besides their important role in biotechnology, these reactions have been discussed as potential virulence factors in pathogenic bacteria and fungi. Other studies have demonstrated the ability of *M. furfur* to release fatty acids from different lipids (Catterall *et al.*, 1978; Hammer & Riley, 2000; Mancianti *et al.*, 2001). Ran *et al.* (1993) did not detect lipases in the supernatant, but determined that the main lipolytic activity was in the insoluble fraction of cell extracts. Contrary to these results, Plotkin *et al.* (1996) demonstrated lipolytic activity in the supernatant and in intracellular soluble and insoluble extracts of *M. furfur*, and further characterized three different lipolytic activities in the soluble fraction.

Abbreviations: CDS, coding sequence; GPI, glycosylphosphatidylinositol; pNP, *p*-nitrophenol; pNPP, *p*-nitrophenylpalmitate; RACE, rapid amplification of cDNA ends.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is DQ155666.

Since virtually no molecular tools are available for *M. furfur* and standard molecular technologies have rarely been applied to this opportunistic pathogen, no attempts have been made so far to elucidate the molecular basis of the extracellular lipolytic activity. In this study, we began to use molecular technologies such as cDNA subtraction, genomic library screening and rapid amplification of cDNA ends (RACE) to discover genes encoding potential virulence factors of *M. furfur*. We cloned and characterized *MfLIP1* encoding the first described secreted lipase of *M. furfur*. Using various recombinant gene products of *MfLIP1*, we show that this gene does encode an extracellular lipase of *M. furfur* and we have begun to analyse the biochemical properties of this enzyme.

METHODS

Strains and media. *Malassezia furfur* strain CBS1878 was used in all experiments. It was cultured either on mDixon plates (Guillot *et al.*, 1996) or in YPD liquid medium supplemented with Tween 80 (1% yeast extract, 1% peptone, 2% glucose, 1% Tween 80). For recombinant plasmids, *Escherichia coli* DH5 α was used as host. Bacterial cells were grown in Luria-Bertani (LB) broth or on LB agar, supplemented with 100 μ g ampicillin ml⁻¹ for selection purposes.

Nucleic acid methods. DNA isolation, RNA isolation, Southern blotting and PCR was performed according to standard protocols (Sambrook & Russell, 2000). Low stringency conditions were used for Southern blots (hybridization at room temperature instead of 42 °C and wash at 40 °C instead of 68 °C) to identify genes similar to *MfLIP1* in the genome of *M. furfur*. For the construction of the genomic library, DNA was extracted with a urea lysis method as described by Gupta *et al.* (2000) and Sansinforiano *et al.* (1998) to obtain sufficiently large fragments. For all sequencing reactions, the BigDye Cycle Sequencing Kit (Applied Biosystems) was used.

cDNA subtraction and RACE. The PCR-select cDNA Subtraction Kit (BD Biosciences Clontech) was used for cDNA subtractions as described in the manufacturer's handbook. cDNA fragments were cloned into pCR2.1 using the TOPO TA Cloning Kit (Invitrogen) and sequenced.

Full-length cDNA sequences were obtained by RACE using the SMART RACE Amplification Kit (Clontech) with primers derived from the sequences obtained by the cDNA subtraction (*MfLip* fwd, 5'-CTC-TAGATTATGACAATCCCCGACAAA-3'; *MfLip* rev, 5'-CTCTAG-AAACACATCCTTCCCTCTGGT-3'). Full-length cDNA of *MfCAT1* was gained via the 3'-RACE procedure alone. For this, the primer *MfCAT1*-RACE was used (5'-ATGGGAAGACTCTTCTTGTCTTC-TTGC-3').

All RACE products were cloned into pCR2.1 via TOPO TA cloning and sequenced using the M13 fwd/rev primer pair (Invitrogen).

Construction of the genomic library. *M. furfur* genomic DNA was partially digested with *Mbo*I and fragments >4 kbp were isolated by agarose gel purification. Fragments were ligated into dephosphorylated, *Bam*HI-digested pBlueScriptII SK(+) (Fermentas) and used for transformation of *E. coli*. Transformants were then screened by colony hybridization with digoxigenin-labelled cDNA fragments as probes. Positive clones were further analysed with PCR using primers *MfLip* fwd and *MfLip* rev (see above), and *MfLip*1

fwd (5'-CCATCGATGCTTCTCTCTTT-3') and *MfLip*1 rev (5'-TCAGGCATTAGAAATCGTAGAC-3') spanning the entire coding sequence.

In silico analysis of DNA and protein sequences. For DNA alignment and homology searches, the NCBI GenBank BLAST server 2.2.10 was used (www.ncbi.nlm.nih.gov/blast/) (Altschul *et al.*, 1990). For protein motifs and predicted functions, the following tools were used: signal peptides were predicted using SignalP 3.0 (Bendtsen *et al.*, 2004) at the CBS prediction server (www.cbs.dtu.dk/services/); glycosylphosphatidylinositol (GPI) modification sites were predicted with the big-PI fungal prediction server (http://mendel.imp.univie.ac.at/gpi/fungi_server.html) (Eisenhaber *et al.*, 1998, 2004); trans-membrane domain prediction and protein localization were performed with the TMHMM 2.0 algorithm (Krogh *et al.*, 2001) and TargetP 1.1 (Emanuelsson *et al.*, 2000) at the CBS prediction server, or with ProtComp 6.0 at www.softberry.com, with the LOCSVMPSI 1.3 server (bioinformatics.ustc.edu.cn) (Xie *et al.*, 2005) or with PA-SUB (www.cs.ualberta.ca/~bioinfo/PA/Sub/) (Lu *et al.*, 2004).

DNA sequences obtained from the RACE reactions and the genomic library clones were assembled using the DNASTAR software package (version 6.0).

Heterologous expression of *MfLip1*. The Pichia Expression Kit (Invitrogen) was used for heterologous expression of *MfLip1*. *MfLIP1* was PCR-amplified from genomic DNA of *M. furfur* using primers *MfLip1*Pic-fwd (5'-ACCATGCCATCGATGCTTCTCTC-3') and *MfLip1*Pic-rev (5'-TCAGGCATTAGAAATCGTAGACACG-3') to amplify the entire coding sequence, including the stop codon and the N-terminal sequences encoding a putative signal peptide (1467 bp). *MfLip1*Pic Δ S-fwd (5'-GATCGAATTCACCATGGTGCTGAAACGT-GGAAAT-3') and *MfLip1*Pic Δ S-rev (5'-CGGCGGCCGCTCAGGCC-ATTAGAAATCGTAG-3') were used to amplify the coding sequence without the signal peptide sequences (1389 bp). The PCR products were cloned into pCR2.1, excised using *Bam*HI and *Not*I and sub-cloned into plasmid pPIC3.5 (Invitrogen) to give pPIC3.5*MfLip1* and pPIC3.5*MfLip1* Δ Sig. pPIC3.5 does not provide sequences for a signal peptide and is normally used for intracellular expression of proteins. pPIC3.5*MfLip1*, pPIC3.5*MfLip1* Δ Sig and the empty vector pPIC3.5 were transformed into *P. pastoris* and transformants were screened for integration of the plasmids by PCR and Southern blotting. Expression of *MfLIP1* was induced by the addition of methanol to minimal medium, as described by the manufacturer (Invitrogen). Supernatants and cell pellets of cultures with integrated plasmids were analysed after induction for the presence of additional protein bands by SDS-PAGE and for lipolytic activity using the lipase activity assay described below. Supernatants containing heterologously expressed *MfLip1* were concentrated tenfold with Microcon centrifugal filter devices (Amicon) with a molecular mass cut-off of 30 kDa.

Lipase assays. Lipase activity was measured using an assay based on hydrolysis of *p*-nitrophenylpalmitate (pNPP). Release of *p*-nitrophenol (pNP) from pNPP (Sigma) was measured in an ELISA reader as the increase in absorption at 405 nm. The assay mixture consisted of 100 μ l 1% Triton-X, 10 mM phosphate buffer, pH 6.0, 1 mM pNPP and 1 μ l concentrated culture supernatant. pNPP was first dissolved in 2-propanol at a concentration of 10 mM because of its low solubility in water. The assay was performed for 1 h at 37 °C, unless stated otherwise. To stabilize the pH-dependent dye pNP, 2 vols 1 M Tris buffer (pH 8.0) was added to adjust the pH before the measurement of the absorbance. For determination of the lipase pH optimum, the assay was performed with phosphate (pH 3.7–5.6) or acetate (pH 5.6–8.0) buffers. To test the effect of metal ions on the activity of the lipase, ions were added to the reaction mixture as 100 mM solutions of NaCl, KCl, CaCl₂, MnCl₂, FeSO₄, ZnCl₂, MgCl₂, NiSO₄, FeCl₃ or CuCl₂.

Activity of the lipase against different Tween or phospholipid substrates was analysed by measuring the release of free fatty acids in a colorimetric assay (Hoffmann *et al.*, 1986). For this purpose, the NEFA-C kit (Wako Chemicals) was used according to the manufacturer's instructions with minor modifications. For the phospholipase assay, the assay mixture consisted of 6 mM phospholipase substrate, 1% Triton-X and 20 mM phosphate buffer, pH 6.0. All phospholipase substrates were sonicated three times for 20 s prior to use to dissolve them completely. Of this mixture, 25 µl was used for each 25 µl of culture supernatant to be tested and incubated at 37 °C. After 6 h incubation, 10 µl was used for the colorimetric reaction in microtitre plates, according to the manufacturer's instructions, and OD₅₃₅ was measured in an ELISA reader. The hydrolysis of Tweens was determined by using the same protocol, with 10 mM Tween replacing the phospholipase substrates.

Statistical analysis. For the statistical analysis, we applied *t*-tests on experiments performed in at least triplicate to determine the statistical significance. Prior to these *t*-tests, a Fisher test was conducted to determine if the variances of the samples differed. If this was the case ($\alpha < 5\%$), a two-sample *t*-test assuming unequal variances had to be performed, otherwise a two-sample *t*-test assuming equal variances was used. A statistically significant difference was assumed for *t*-tests $\alpha < 5\%$.

RESULTS

Sequence of MfLIP1

Cells of *M. furfur* were grown in lipid-containing medium, the mRNA was isolated, transcribed into cDNA and a cDNA subtraction protocol was used to identify genes associated with growth in the medium (data not shown). One clone contained a cDNA fragment which had high sequence similarity to known extracellular lipases, particularly to members of the *Candida albicans* lipase gene family (Hube *et al.*, 2000). To obtain the entire cDNA sequence of the fragment we used a RACE protocol for both the 5' and 3' directions. Using this protocol, we obtained one PCR fragment for each direction, with an overlap long enough to allow the combination of the two sequences. The combined full-length cDNA consists of 1765 bp, including sequences with putative 5' and 3' untranslated regions and a coding sequence (CDS) of 1464 bp.

This CDS encodes a protein of 488 aa with a predicted molecular mass of 54.3 kDa. Sequence analysis identified a conserved serine residue within the GX SXG motif typical for lipases (Derewenda & Derewenda, 1991; Derewenda, 1994), and a histidine residue in a position aligning with the conserved histidine of other lipases (Hube *et al.*, 2000) (Fig. 1). Additionally, the deduced protein sequence has an overall high sequence similarity to extracellular lipases. A BLAST search against the NCBI GenBank database revealed that the protein sequence shares 33% identity and 51% similarity with a lipase of *Arxula adeninivorans* and 32% identity and 48% similarity with Lip2 of *C. albicans* (Figs 1 and 2). Therefore, we predicted that the CDS encodes a lipase which we named MfLip1 (*M. furfur* lipase 1).

In silico analysis using SignalP 3.0 (Bendtsen *et al.*, 2004) identified a signal peptide of 26 aa at the N terminus of

MfLip1, suggesting that the protein is directed into the secretory pathway. Since no transmembrane domains or conserved GPI-anchor motifs were detected, we assumed that MfLip1 may either be located within the secretory pathway, guided to the vacuole or secreted into the extracellular space (Lee *et al.*, 2003). This assumption was supported by TargetP, which predicts that the protein enters the secretory pathway with 95.4% probability.

Analysing the gene locus of MfLIP1

Since the genome sequence of *M. furfur* is unknown we constructed a genomic library to identify the genomic sequence of MfLIP1 and the flanking genes of the native locus.

The library was probed with the original cDNA fragment and one positive clone was identified. PCR with primers deduced from the cDNA revealed that this clone, with an insert of >6 kb, contained the entire coding sequence of MfLIP1. The insert was partially sequenced by primer walking.

Sequence analysis of the clone and alignment with the cDNA sequence revealed that MfLIP1 does not contain any introns. Two more genes were identified in the direct neighbourhood of MfLIP1 (Fig. 1). Downstream (1591 bp) of the stop codon of MfLIP1 we identified a putative gene encoding a protein with high similarity to copper amine oxidases (not shown). *In silico* analysis with ProtComp and LOC SVMPSI predicted that this protein resides in the peroxisome.

Another putative ORF was identified 1025 bp upstream of MfLIP1 with high similarities to bacterial catalases (62% identity and 77% similarity at the amino acid level to a catalase of *Paracoccus denitrificans*) and therefore termed MfCAT1. The coding sequence on the genomic library plasmid was found to be incomplete on the 3'-side, so the 3'-RACE protocol was used to obtain the cDNA sequence of MfCAT1. This sequence information was then used to PCR-amplify and sequence the entire CDS from *M. furfur* genomic DNA.

SignalP predicts that the first 19 aa of the N terminus of MfCat1 encode a signal peptide for transport into the endoplasmic reticulum. According to TargetP, the probability of mitochondrial localization of the catalase is only 2.2%, whereas the probability that it enters the secretory pathway is 96.4%. Similar to MfLip1, no C-terminal GPI-anchor site could be detected by big-PI 3.1. Analysis with PA-SUB suggests that MfCat1 is not guided to the vacuole (0.0%) but, like MfLip1, is secreted to the extracellular space (90.6%). No gene similar to MfCAT1 was found in the genome of the closely related plant pathogen *Ustilago maydis*.

MfLIP1 is not a member of a lipase gene family in *M. furfur*

Growth of *M. furfur* is lipid-dependent; therefore, this fungus must contain one or more lipases which enter the

Fig. 1. Schematic overview of the genomic locus of *MfLIP1*. The sequence was derived from the genomic library clone containing *MfLIP1*. At the top, the recognition sites of five common restriction endonucleases are shown. Three proteins were predicted to be encoded by this stretch of DNA (indicated by arrows): *MfLip1*, a putative secreted catalase (*MfCat1*) and an amine oxidase. Signal peptides (SP) are indicated by white boxes. The representation of *MfLip1* is expanded to illustrate the positions of some conserved amino acids. These are the serine and the histidine of the catalytic triad typical of lipases, with the position of the aspartate uncertain, and four cysteine residues, which align with known conserved cysteines in the *C. albicans* lipase gene family. Additionally, parts of an alignment of *MfLip1* and the proteins with the highest BLAST scores are shown in detail. The source organisms are indicated in parentheses: *M. furfur* (Mf), *Aspergillus nidulans* (An), *Arxula adenivorans* (Aa), *Debaryomyces hansenii* (Dh) and *C. albicans* (Ca). Abbreviations: hyp. prot., hypothetical protein; lip., lipase; prot. prod., protein product.

extracellular space. Since it had been shown that other pathogenic fungi such as *C. albicans* contain multiple lipase genes (Hube *et al.*, 2000), we screened for further lipase genes in the genome of *M. furfur*. Southern blot analysis with the original *MfLIP1* cDNA fragment as a probe revealed only faint additional bands in addition to fragments containing *MfLIP1* even under low stringency conditions (Fig. 3). We concluded that *MfLIP1* is not a member of a gene family with similar lipase genes in *M. furfur*.

We also questioned whether genes similar to *MfLIP1* exist in species related to *M. furfur* and probed the genomes of seven *Malassezia* species with *MfLIP1* by low stringency Southern blot analysis. For *M. dermatitis*, *M. globosa*, *M. obtusa*, *M. restricta*, *M. slooffiae* and *M. sympodialis*, no signals were detected. Only with *M. pachydermatis* genomic DNA were two bands visible (not shown).

Heterologous expression of *MfLip1* in *P. pastoris*

The deduced sequence of *MfLip1* showed both similarity to known extracellular lipases with conserved lipase consensus sequences and a putative signal peptide. To confirm that

MfLip1 does in fact have lipolytic activities, and that the signal peptide is able to direct the enzyme to the extracellular space, the protein was heterologously expressed in the yeast *P. pastoris*.

To show that the signal peptide is essential for secretion, two different plasmids for expression in *P. pastoris* were constructed. The first plasmid contained the complete ORF, including the predicted *M. furfur* signal peptide. The second construct contained a truncated version without the signal peptide sequence. The vectors were used for transformation of *P. pastoris* and transformants were screened for additional proteins and lipolytic activity in the supernatant (compared to activity in the cell pellet) after 5 days of induction.

In the supernatant of the *P. pastoris* strain harbouring the complete ORF, a distinct band of approximately 55 kDa was detected (Fig. 4). In contrast, the supernatant of the strain containing the truncated version showed no additional bands compared to the *P. pastoris* wild-type strain. Therefore, the putative signal peptide is able to direct the protein into the supernatant. This was confirmed in a lipase activity assay. Strong lipolytic activity was measured in the

Fig. 2. Dendrogram based on the protein sequence of MfLip1 and other known and putative lipases. The highest scoring lipases from a BLAST protein similarity search with MfLip1 were combined in a phylogenetic tree and the tree was rooted with the mycobacterial lipases as an outgroup. The *Candida* lipases cluster together as expected, as well as the lipases from *Gibberella zeae*. MfLip1 is most closely clustered with lipases from *Aspergillus* species and predicted proteins from *Ustilago maydis*, a closely related plant pathogen.

supernatant of the transformant harbouring the complete ORF, whereas no such activity was shown for the strain with the truncated version or the wild-type.

pH and temperature optimum of MfLip1

To determine the biochemical properties of the discovered lipase, the lipase was concentrated and the influence of the pH and temperature on the lipolytic activity was examined. In phosphate/acetate-buffered assays, MfLip1 showed a peak of activity at about pH 5.8 with a more than threefold reduction around pH 4.0 and a smaller drop to about 65 % activity at pH 7.0. Measurements in the range of pH 8.0 and above produced no reliable results because of the instability of the substrate under these conditions.

The temperature activity curve showed a steady increase in activity from 30 % at room temperature (20 °C) to a maximum at about 40 °C. Above 40 °C the activity dropped rapidly, resulting in a residual activity of 12 % at 50 °C, the highest temperature investigated. In addition, esterase activity was detected for MfLip1 using the four-carbon chain substrate *p*-nitrophenylbutyrate. Esterolysis catalysed by MfLip1 had similar characteristics to lipolysis of pNPP, but

reached only half of the maximum lipase activity under the conditions used.

Effect of different metal ions on MfLip1 activity

Metal ions are known to modify the activity of lipases, either enhancing or interfering with the rate of hydrolysis. To investigate such effects on MfLip1, different metal ions were added to the reaction mixture. All divalent ions inhibited the lipolytic activity to different extents at concentrations of 0.1–10 mM, with 10 mM Fe^{2+} having the strongest effect (99 % reduction), followed by Fe^{3+} (96 %) and Ca^{2+} (95 %). Cu^{2+} , Fe^{2+} , Fe^{3+} and Zn^{2+} all had a strong effect even at 0.1 mM (Table 1). The monovalent ions Na^+ and K^+ exhibited less dramatic inhibition of the lipase in the higher concentration range (35 and 45 %, respectively, at 10 mM) and no statistically significant reduction in lipase activity at 0.1 mM.

Other substrates of MfLip1

In standard culture medium, different Tweens are usually used for the provision of lipids. The hydrolysis of different Tween compounds by MfLip1 was tested by measuring the

Fig. 3. *MfLIP1* is not part of a family of highly similar genes in *M. furfur*. *MfLIP1* was used as a probe in a Southern blot with different restriction digests of *M. furfur* genomic DNA. Even under low stringency conditions, as shown here, only very weak bands were detected in addition to the expected bands of *MfLIP1*. Genomic DNA was digested with *Bam*HI (lane 2), *Eco*RI (3), *Hind*III (4), *Kpn*I (5), *Pst*I (6) and *Xba*I (7). For *Eco*RI and *Pst*I, two bands were expected, since the enzymes cut once in the coding sequence. All other enzymes have no recognition sites in *MfLIP1*. The linearized plasmid bearing the original cDNA fragments was used as a positive control in lane 8. Markers in lane 1 are DIG marker II (Roche) and in lane 9 DIG marker III. Marker band sizes are given in base pairs.

release of free fatty acids from 10 mM substrates. *MfLip1* exhibited lipolytic activity against all three Tween types tested (Tween 20, 40 and 80), with Tween 80 being the best substrate (Fig. 5).

In addition to lipase and esterase activities, some lipases may also show phospholipase activity. Using phospholipase and lysophospholipase activity tests, *MfLip1* exhibited no such activities (data not shown), whereas free fatty acids were released from monoacylglycerol (Fig. 5) and triacylglycerol (data not shown), showing that *MfLip1* is in fact a lipase.

DISCUSSION

Since lipids are essential for growth of most species of the genus *Malassezia*, it must be concluded that these fungi are able to hydrolyse lipids extracellularly. Furthermore, lipolytic activity has been associated with survival and pathogenicity of certain members of this genus such as *M. furfur*. Here we report for the first time the identification and characterization of a gene encoding an extracellular lipase of this opportunistic fungus.

M. furfur has been poorly investigated at the molecular level. So far, the sequences of only eight cDNAs encoding proteins of *M. furfur* have been deposited in the GenBank database, and most of these sequences describe proteins with potential roles as allergens. Only partial sequences of two genes, encoding a mitochondrial cytochrome *b* (Biswas *et al.*, 2001) and a chitin synthase 2 (Kano *et al.*, 1999), have yet been described with functions defined according to

Fig. 4. The putative signal peptide of *MfLip1* directs the protein into the supernatant. The supernatants of *P. pastoris* transformants bearing the empty vector (pPIC3.5), the complete *MfLIP1* ORF (*MfLIP1*) or a truncated version without the signal peptide (*MfLIP1*ΔSig1 and 2) were analysed in a Coomassie-blue-stained SDS-PAGE gel for the presence of protein (a) and with a pNPP lipase assay for lipolytic activity (b). A distinct additional band of about 50–60 kDa (the predicted size of *MfLip1*) can be seen in the supernatant of transformants containing the putative signal peptide, which is not present in the other transformants (a). Accordingly, lipolytic activity in the supernatant could only be detected for clones with the complete ORF (b).

Table 1. Inhibition of lipase activity by different metal ions in a pNPP assay with heterologously expressed *MfLip1*

Metal ion	Remaining lipase activity* at metal ion concentration of:		
	10 mM	1 mM	0.1 mM
Ca ²⁺	4.9 ± 1.3	12.7 ± 0.5	52.1 ± 9.7
Cu ²⁺	27.4 ± 1.9	25.7 ± 1.8	21.1 ± 3.2
Fe ²⁺	1.0 ± 3.1	7.5 ± 0.1	26.3 ± 3.4
Fe ³⁺	3.9 ± 1.0	42.9 ± 3.4	30.8 ± 4.1
K ⁺	54.5 ± 2.0	64.4 ± 9.6	88.4 ± 9.3†
Mg ²⁺	26.4 ± 3.8	43.3 ± 0.4	75.2 ± 10.1
Mn ²⁺	9.2 ± 0.6	23.1 ± 2.9	65.9 ± 11.0
Na ⁺	65.0 ± 1.9	84.0 ± 3.7	97.2 ± 8.4†
Ni ²⁺	10.8 ± 0.6	22.4 ± 2.9	61.7 ± 7.1
Zn ²⁺	41.2 ± 1.4	20.7 ± 1.8	37.3 ± 2.2
H ₂ O		100.0 ± 2.3	

*Values are given as mean percentage ± SD of four samples compared to a sample containing no additional ions (H₂O).

†All residual activities were significantly different from the H₂O reference with the exception of the values marked with †.

Fig. 5. Different Tweens are hydrolysed by MfLip1. Tweens 20, 40 and 80 and 1-monopalmitoylglycerol (MPG) (10 mM each) were incubated together with concentrated MfLip1 (grey bars) or *P. pastoris* (transformed with the empty vector) (black bars) supernatant in a NEFA-C assay. After 6 h, the amount of released free fatty acids was determined (representative sample showing means of three replicates $\pm$ SD). MfLip1 demonstrates hydrolytic activity against all Tween compounds and monoacylglycerol, whereas for *P. pastoris* no such activity could be shown. The highest rate of lipolysis was measured with MPG and Tween 80 and the lowest with Tween 20.

homology. In this study, we constructed and screened a genomic library and used RACE to identify and clone *MfLIP1*. Strong similarities of the deduced sequence of *MfLIP1* with known lipase sequences, the existence of conserved lipase motifs and a prototypic N-terminal signal peptide sequence suggest that *MfLIP1* encodes a secreted lipase. By comparing the genomic DNA with the cDNA sequence we showed that *MfLIP1* does not contain introns, as is frequently observed in basidiomycetes such as *Ustilago maydis* or *Cryptococcus neoformans*. By analysing the genomic locus of this putative lipase gene, we showed that *MfLIP1* is flanked by two other genes, encoding a putative amine oxidase and, interestingly, a catalase with a putative signal sequence. Therefore, it may be possible that this catalase is secreted.

Both lipase and catalase activity are commonly used for differentiation of *Malassezia* species (Gueho *et al.*, 1996; Mayser *et al.*, 1997). With the sequence of both genes available, it is now possible to use *MfLIP1* and *MfCAT1* for taxonomic and diagnostic purposes. Southern analysis showed that the lipase gene appears to have no homologues in most *Malassezia* species, except *M. pachydermatis*.

By using the heterologous *P. pastoris* expression system and different constructs containing either the entire ORF of *MfLIP1* or a truncated version lacking the signal peptide, we showed that MfLip1 is a secreted lipase.

Biochemical data of the heterologously expressed lipase seem to be in accordance with the lifestyle of *M. furfur*. The pH optimum of 5.8 fits well with the normal pH of the skin (5.4–5.9), and the temperature optimum of 40 °C is only slightly higher than the body temperature; the main habitats

of *M. furfur* are on seborrhoeic skin and inside the sebaceous gland of the hair follicle. It should also be noted that the lipase activity retains about 95 % of its peak value at 37 °C.

Although it is clear that a lipolytic activity is essential for *M. furfur*, the definite role of MfLip1 in the life cycle of *M. furfur* is not clear. The results presented here strongly suggest that the lipase functions in the acquisition of free fatty acids, especially since the hydrolysis of Tween compounds by MfLip1 was demonstrated. These artificial lipids are used in standard culture media for *M. furfur* and are both necessary and sufficient as a C-source. Therefore, MfLip1 may play an important role in the growth of *M. furfur* in culture media or within its natural environment.

However, it seems that lipolytic activity has more roles than simply providing fatty acids for cell growth. Cell-wall-associated lipids have been shown to be involved in the suppression of the proinflammatory immune response (Kesavan *et al.*, 2000) and the avoidance of internalization and killing by phagocytic cells (Ashbee & Evans, 2002; Gueho *et al.*, 1998). Furthermore, these lipids are thought to participate in the adhesion of *M. furfur* to the host cell via hydrophobic interactions (Faergemann *et al.*, 1983; Mittag, 1995).

Since *M. furfur* is not able to synthesize lipids for the outermost cell wall layer *de novo* (Shifrine & Marr, 1963), the constituent fatty acids are recruited from the environment. This is reflected, for example, by the fact that its composition changes according to the lipids encountered in the habitat (Caprilli *et al.*, 1973; Mittag, 1995). Accordingly, secreted lipases most probably contribute to the formation of this protective lipid layer, pointing to a possible role of MfLip1 or other extracellular lipases in pathogenesis. Definite proof can only be achieved by producing a MfLip1 knockout mutant. However, a transformation protocol has not yet been described and it is likely that this may be a difficult task because of the comparatively thick cell wall, making up 26–37 % of total cell volume, and the unusually high lipid content of about 15 %. These characteristics are possibly responsible for the fact that standard transformation protocols fail for *M. furfur* (not shown).

In summary, we have cloned and characterized *MfLIP1*, encoding the first putative secreted lipase of *M. furfur*. By using a recombinant gene product of *MfLIP1*, we have shown that this gene in fact encodes an extracellular lipase and we have begun to analyse the biochemical properties of this enzyme. Our data suggest that MfLip1 may contribute to the extracellular lipolytic activity of *M. furfur* *in vitro* and *in vivo*.

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