Multiple Hydrophobin Genes in Mushroom

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Hydrophobins are small secreted fungal proteins that form amphipathic films on the hyphal surfaces. In the wood-rotting fungus *Schizophyllum commune*, four different hydrophobins are known with well established functions during vegetative growth and fruiting body development. Our study aims at elucidating the role of these proteins in wood penetration and lignocellulose degradation. Blast searches of the genome of the dung fungus *Coprinopsis cinerea* revealed a surprising number of 34 different hydrophobin genes in this species. Functional analysis of these genes is in progress.

1. Introduction

Schizophyllum commune, the Split-Gill Mushroom, is one of the most widely distributed wood inhabiting basidiomycete found throughout the tropical and temperate regions (Raper et al. 1958, James et al. 1999, James and Vilgalys 2001). The species is regarded as mild rot in temperate regions and severe wood destroyer in the tropical regions (Schmidt and Liese 1980). Most commonly, *S. commune* is found in nature on fallen trunks and branches of deciduous trees (Fig. 1, left), less often on wood of conifers (Cooke 1961, Breitenbach and Kränzlin 1991). Moreover, the fungus can act as a pathogen on standing trees (Latham 1970, Adaskaveg et al. 1993; Fig. 1, middle and right).

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Figure 1. *Schizophyllum commune* fruiting bodies found on a fallen beech tree in the Billingshäuser Schlucht, Göttingen (left; May 2004) and on a living *Juglans ailantifolia* tree growing next to the GZMB (Göttinger Zentrum für Molekularbiologie) on the ground of the Georg-August-University Göttingen (middle; November 2004). Note that in spring 2005 the tree produced leaves on all healthy branches but not on branches infected with *S. commune* (right; May 2005)

S. commune is usually considered to be a white rot fungus. However, wood decay tests in the laboratory in most instances did not result in considerable weight losses (Hegarty et al. 1987, Nsolomo et al. 2000, Humar et al. 2001, 2002, Schirp et al. 2003). Schmidt and Liese (1980), Nilsson and Daniel (1983) and Leithoff and Peek (2001) measured between 1 to 6% weight loss for different strains and concluded the fungus is a rather weak wood-destroyer. In rare cases, mass losses of nearly 50% were observed mainly due to lignin degradation and to a low degree due to cellulose degradation (Hong 1982). Although there might be no weight loss in wood decay tests, toughness of wood can be negatively affected. 32% strength loss had been measured (Schirp et al. 2003). In flake board tests with *S. commune*, weight loss of 9.6% occurred together with strength loss (67.5% modulus of rupture loss; Hadi et al. 1995). A monokaryon of *S. commune* was shown to some extent to demethylate lignin, but this was not due to production of laccases, peroxidases or ligninases (Trojanowski et al. 1986). Poor lignin degradation in another study was thought to result from the inability of the fungus to solubilize lignin (Boyle et al. 1992). On wheat straw, some tropical isolates were shown to degrade lignin (up to 15%) and to cause simultaneous mass loss of up to 23.5%. Another strain showed even higher mass loss (26.7%) but no lignin decay (Capelari and Zadrazil 1997). Lignin in olive pomace was efficiently degraded by a *S. commune* isolate (up to 52.7% of lignin breakdown). During pomace degradation, high laccase activities were recorded (Haddadin et al.

2002). Many *S. commune* strains have xylanase and cellulose activities (Schmidt and Liese 1980, Clarke and Yaguchi 1986, Hegarty et al. 1987, Bray and Clarke 1995, Haltrich et al. 1995, Thygesen et al. 2003) and most of them have also laccase and peroxidase activities (Schmidt and Liese 1980). de Vries et al. (1986) described laccase activity specific to the dikaryotic state of *S. commune* whilst in the parental monokaryons, they could not detect this activity. Phenoloxidase activities were not found in other studies, also not upon treatment with phenolic inducers (Boyle et al. 1992, Nsolomo et al. 2000). By the well established xylanase and cellulose activities it has been suggested that the main role in nature is to recycle carbon by breaking down cellulose and xylans in fallen wood (Raper and Fowler 2004). In conclusion, the ability of *S. commune* to degrade wood remains to be a puzzle.

S. commune is used as a model fungus to study mating types and fungal development in basidiomycetes (Raper and Fowler 2004). Well known is the discovery of hydrophobins in this fungus, small-secreted fungal proteins of about 100 to 140 amino acids with 8 conserved cysteine residues. Upon secretion, hydrophobins self-assemble into amphipathic stable films that cover fungal cells and make their surfaces hydrophobic. Therefore, these films enable fungal structures to grow into the air and protect them from adverse environmental conditions. Due to the hydrophobin coating, aerial hyphae are repellent towards water (Fig. 2). Within mushrooms, hydrophobin films coat the air channels and prevent them from filling with water. In total, four different hydrophobins have been described in *S. commune*. SC3, the best characterized hydrophobin, is expressed in the vegetative mycelium of monokaryons and dikaryons. The other three, SC1, SC4 and SC6 are dikaryon specific. SC4 is low expressed in the mycelium and well in mushroom tissues. SC1 and SC6 are specific to fruiting stages (Wösten 2001, Walser et al. 2003).

It is not known whether hydrophobins participate also in wood colonization and pathogenicity of *S. commune* and in degradation of lignin as a hydrophobic component (Wösten et al. 1994). Is it necessary that the hyphae have a hydrophobic surface when growing in wood? Hyphae of the species have been shown to grow within the lumen of tracheids and vessels and attack the wood by loosening the S_3 layer from the rest of the wood cell walls before localized dissolution of cell wall substance results in narrow slits within the S_2 layer. Pronounced lamellation of the S_2 layer occurs in later stages of degradation and only then hyphae were found in the slits of the S_2 layer (Nilsson and Daniel 1983).

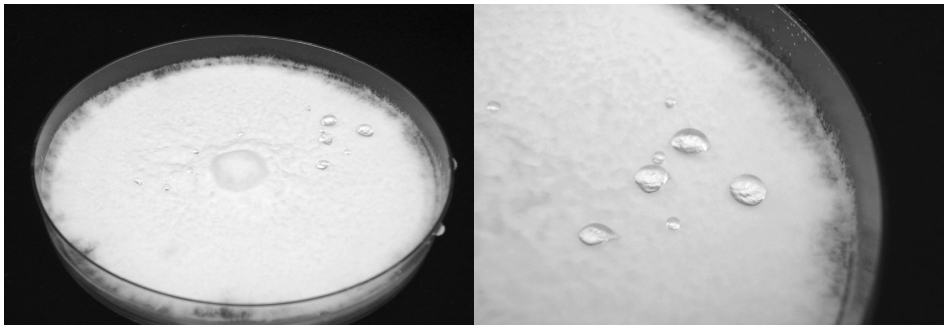

Figure 2. Aerial mycelium of *S. commune* monokaryon 4-39 is water-repellent due to the amphipathic SC3 film covering the surfaces of the hyphae

Another model fungus for studying development in basidiomycetes is the dung fungus *Coprinopsis cinerea* (Kües 2000, Kües et al. 2002, 2004). So far, only one hydrophobin (CoH1; Fig. 3) has been described in this species. CoH1 is expressed in the vegetative mycelium of monokaryons and, less efficient, of dikaryons. Although shorter in length than the *S. commune* hydrophobin SC3, the hydropathy plots of the two proteins are very similar (Fig. 4). A gene for another potential hydrophobin (CoH2) had been detected by sequencing on a 10 kb DNA fragment carrying gene *coH1* (Ásgeirsdóttir et al. 1997). Studies in our lab aim at further understanding roles of *S. commune* and *C. cinerea* hydrophobins in growth and development.

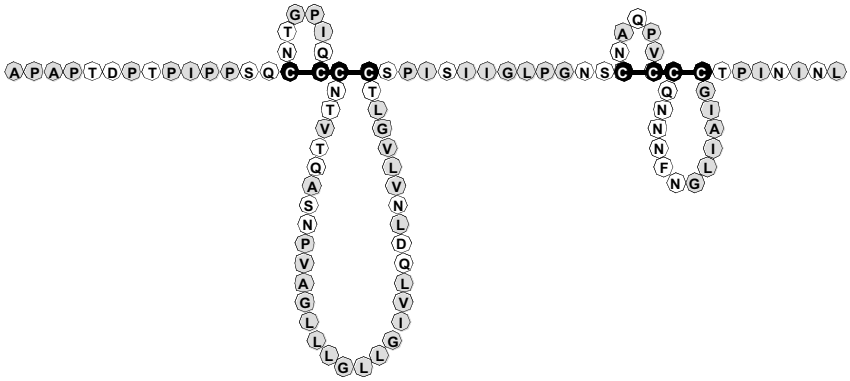

Figure 3. Model of *C. cinerea* hydrophobin CoH1 analogous to a *S. commune* SC3 model presented by Wessels (2000). The eight conserved cysteines (shown as white letters in filled black circles) are thought to interact to give the typical hydrophobin folding

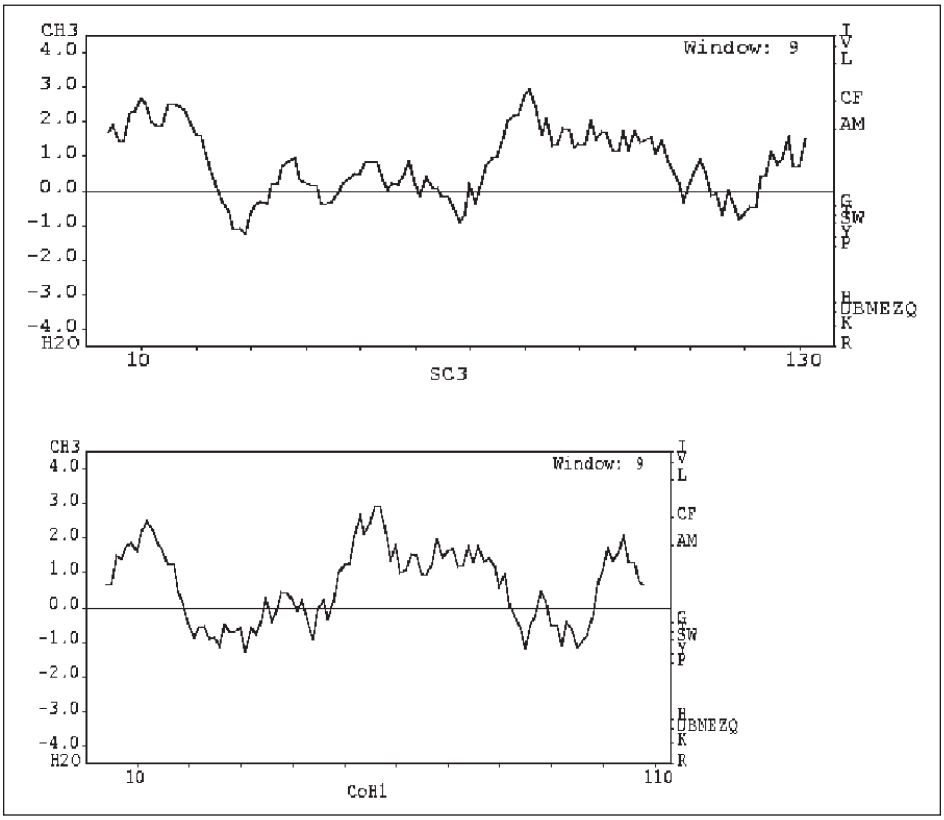

Figure 4. Hydropathy plots for *S. commune* hydrophobin SC3 (top) and *C. cinerea* hydrophobin CoH1 (bottom) calculated according to Kyte and Doolittle (1982)

2. Materials and Methods

2.1. *Strains, culture conditions and light and FTIR microscopy*

S. commune monokaryon 4-39 (*A41 B41*) was kindly supplied by Han A.B. Wösten. To infest wood, the strain was grown at 25°C in light on minimal medium (Dons et al. 1979). Sterilized beech wood blocks (3 x 1 x 0.5 cm) were placed onto 1 mm thick stainless steal grids layed on established mycelium to avoid direct contact between the wood and the agar. Wood blocks were incubated with the fungus for 20 weeks before sections of 25 µm

thickness were prepared with a microtome. Sections were transferred onto gelatin-coated glass slides, stained for 10 min with lactophenol blue (Esser 1976), dried at 60°C and cleaned with water. Coverslips were fixed on the samples with Depex (Serva Electrophoresis, GmbH, Heidelberg) and dried overnight with a weight placed on top of the cover slip. Samples were analyzed with a Zeiss Axiophot photomicroscope equipped with a Soft Imaging Color View II digital camera. For FTIR microscopy, mycelium grown on the surface of the wood blocks was air-dried. An FTIR spectrometer Equinox 55 in combination with an IR microscope Hyperion 3000 (Bruker Optics, Ettlingen, Germany) with a single channel MCT detector was used to record FTIR spectra of the mycelium on KBr windows (2 mm) in transmission mode with a 15 x Cassegrain-objective, knife edge aperture of 20 x 45 µm, resolution of 4 cm^{-1}, 16 scans (Naumann et al. 2005).

C. cinerea strain AmutBmut is a self-compatible homokaryon that forms fruiting bodies without prior mating to another strain (Boulianne et al. 2000) under standard fruiting conditions (Granado et al. 1997). Primordia and fruiting bodies were harvested for hydrophobin isolation following the protocol of Ásgeirsdóttir et al (1998). Isolated proteins were separated by SDS-PAGE (Garfin 1990).

3. Results

3.1. *Growth of* Schizophyllum commune *strain 4-39 in beech wood*

In sections of beech wood incubated for 20 weeks with *S. commune* strain 4-39, lactophenol-stained fungal hyphae were visible within vessels (Fig. 5). Signs for attack of the wood by the fungus were not obvious from optical inspection of the samples.

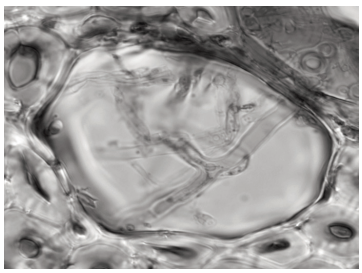

Figure 5. Lactophenol blue-stained hyphae of *Schizophyllum commune* strain 4-39 in a beech wood vessel

Wood and mycelium grown in and on the beech wood in unstained sections was analyzed by Fourier transform infrared microscopy (FTIR) microscopy (Naumann et al. 2005). FTIR microscopy allows local resolution of the chemical composition of a sample. The absorption of infrared light by dipolar molecular bonds of polysaccharides, proteins, lipids, aromatic and many other compounds results in a typical absorption spectrum for the sample (Fig. 6).

Spectra recorded by FTIR microscopy distinguish wood and mycelium from each other (Naumann et al. 2005). Spectra from mycelium grown within vessels are similar to each other (results not shown) as well as spectra from mycelium grown on the wood surface (Fig. 6). The two groups of spectra relate to each other. However, compared to mycelium within vessels, spectra of surface mycelium showed a more pronounced peak at 1640 cm^{-1} (Naumann et al. 2005). This wave length corresponds to the amide I band of peptide bonds (de Vocht et al. 1998). Purified SC3 hydrophobin, the most abundant protein on the aerial hyphae of *S. commune* 4-39 strain (Wessels et al. 1991, de Vries et al. 1993), has a characteristic peak at this wave length (de Vocht et al. 1998).

Next to wildtype strains, there exist *SC3* knockout strains of *S. commune* (van Wetter et al. 1996) that will be very useful in analyzing whether the fungus needs hydrophobins for growth in wood. By comparison, these mutants will be useful to establish in the FTIR spectra of *S. commune* wildtypes specific peaks corresponding to the hydrophobins. Knowing this, it might be possible to deduce whether detected differences in spectra of mycelium grown in and on wood result from differences in hydrophobin expression.

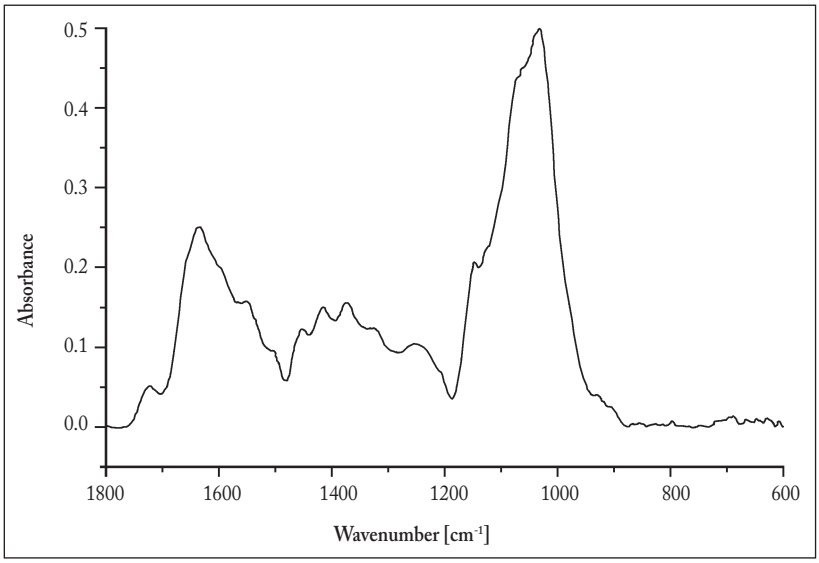

Figure 6. FTIR spectrum of mycelium of *Schizophyllum commune* grown on the surface of wood blocks, recorded with an FTIR microscope with MCT single channel detector. The spectrum has been baseline corrected. The arrow points to 1640 cm^{-1} where hydrophobin SC3 has a characteristic peak.

3.2. *Hydrophobins and hydrophobin genes in* Coprinopsis cinerea

Hydrophobins in *S. commune* and other basidiomycetes have important structural functions in mushroom development (Wösten 2001, Walser et al. 2003). Since in *C. cinerea* nothing was known on hydrophobins in mushroom development, by standard protocols (Ásgeirsdóttir et al. 1998) we tried to isolate such proteins from young fruiting bodies from homokaryon AmutB-mut (stages of rapid stipe elongation and basidiospore pigmentation). However, we were unable to bring hydrophobins properly into solution that were isolated from such late stages in fruiting body development. Such probes were contaminated with melanin that probably was produced for spore blackening in the mushroom maturation process. Since not properly been dissolved, on SDS gels such hydrophobin samples caused smear (Fig. 7). In contrast, we could solubilize hydrophobins isolated from earlier stages of primordia development prior to spore formation and separate them on SDS gels (Fig. 8). Multiple hydrophobin bands were visible ranging in sizes from

10 to 15 kDa, which is the typical size for this class of proteins (Wösten 2001, Walser et al. 2003) with the only exception of *S. commune* SC3 that is a 24 kDa protein (Wösten et al. 1993).

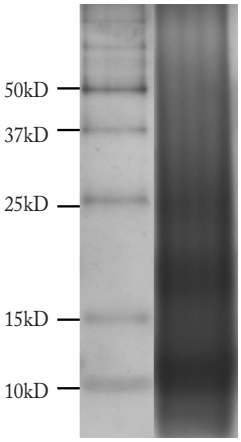

Figure 7. 15% SDS-PAGE showing poorly dissolved hydrophobins from young fruiting bodies of *Coprinus cinerea* homokaryon AmutBmut in a dark smear (right lane). At the left, molecular size marker

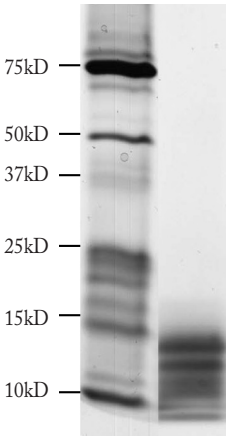

Figure 8. 15% SDS-PAGE showing well dissolved and several well separated hydrophobins from primordia of *Coprinus cinerea* homokaryon AmutBmut (right lane). At the left, molecular size marker.

With the release of the genomic sequence of *C. cinerea* strain Okayama 7 by the Broad Institute (http://www.broad.mit.edu/annotation/fungi/coprinus_cinereus/), we were able to Blast search the genome for hydrophobin genes and found the amazing total number of 34 different genes. All potential gene products contain the eight conserved cysteines involved in protein folding (Fig. 3) and their hydropathy plots resemble those of *S. commune* SC3 and *C. cinerea* CoH1 (Fig. 4). Expression studies of the different *C. cinerea* genes will have to verify whether they are all functional or whether there are pseudogenes amongst them. The protein gel shown in Fig. 8 supports that many of them will be functional.

The situation of multiple genes as in *C. cinerea* appears not be uncommon in higher basidiomycetes since in the genome of the wood-rotting fungus *Phanerochaete chrysosporium* (Martinez et al. 2004), we found 20 different hydrophobin genes by Blast searches. Whilst four different hydrophobin genes are currently known in *S. commune* (Wessels et al. 1995), also this fungus may have many more than is so far evident from experimental work.

4. Conclusion

Basidiomycetes can have many different hydrophobins. Work in *S. commune* showed before that hydrophobins are differently expressed and have been evolved for different development functions (Wösten 2001, Walser et al. 2003). So far, it is not known whether hydrophobins are needed for growth in and degradation of lignocellulosic substrates such like wood and straw. In *C. cinereus*, we found the amazing number of 34 different hydrophobin genes and it will be a demanding task to determine all their functions in growth and development.

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