Hydrophobins: Proteins that Change the Nature of the Fungal Surface

Joseph G. H. Wessels

Department of Plant Biology, Groningen Biomolecular Sciences and Biotechnology Institute, University of Groningen, Kerklaan 30, 9751 NN Haren, The Netherlands

1. INTRODUCTION

Although fungi, with the exception of yeasts, are multicellular, their mechanisms of growth and development are quite distinct from those of plants and animals. In the multicellular fungi, the fundamental growth unit is the hypha, which may or may not be divided into cells. The hypha is essentially a tubular structure containing all the typical eukaryotic cytoplasmic components surrounded by a rigid wall. Hyphae are highly polarized and grow only at one end, where a new wall is

ADVANCES IN MICROBIAL PHYSIOLOGY VOL 38
ISBN 0-12-027738-7

deposited in such a way that the tubular shape is generated, despite the presence of a high internal hydrostatic pressure (turgor) (Wessels, 1986, 1990, 1993a). Hyphae regularly branch and give rise to a mycelium that forms a colony. The colony grows radially at its periphery, where apical extension and branching occur. The mycelium thus colonizes a substrate, maintaining a constant ratio between the total length of hyphae and the number of tips, a ratio known as the "hyphal growth unit" (Trinci, 1974; Trinci *et al.*, 1994). Another useful concept developed by Trinci and co-workers (see Trinci *et al.*, 1994) is that extension of tips of individual hyphae is supported by a certain volume of protoplasm; this mycelial region involved in tip growth is called the "peripheral growth zone" of the colony.

In principle, hyphae appear designed for unlimited transport of water, nutrients and cytoplasmic components. In the zygomycetes the hyphae are not subdivided by septa so that the cytoplasm (with many nuclei) is essentially contained in a continuous branched tubular system, making it difficult in these organisms to apply the concept of cellularity. In the second major group of the fungi, the ascomycetes, septa are present but these contain large pores that do not seem to obstruct movement of organelles such as nuclei and mitochondria. Only in the third group, the basidiomycetes, do septa divide the hyphae into separate compartments. The small septal pore and the elaborate membranous structures (parenthosomes) that cover it (Moore, 1985) effectively prevent passage of nuclei and mitochondria, but apparently do allow for transport of water and nutrients. In fact, members of the basidiomycetes have a great capacity for long-range translocation (Jennings, 1984). However, in situations where nuclei have to be exchanged in these basidiomycetes, as in mating interactions, the septa are dissolved so that nuclei and other cytoplasmic components can move freely through the hyphal tubes (Raper, 1966; Wessels, 1978).

Because hyphae not only grow but also secrete enzymes at their apices (Wösten *et al.*, 1991), the fungal mycelium is ideally suited for growing into solid organic substrates and for degrading the constituent polymers from within (Wessels, 1993a). The colonization is facilitated by the fact that, in the local absence of nutrients, hyphal growth can be sustained by the transport of water and nutrients from a food base (Jennings, 1984, 1994; Rayner *et al.*, 1995). In this way the mycelium can explore large areas that contain only isolated patches of nutrients.

Another manifestation of the ability of the mycelium to transport water and assimilates to hyphae that are unable to acquire nutrients, is the occurrence of emergent growth. For instance, hyphae may give up assimilating nutrients and grow into the air, causing the "mouldy" appearance of many fungi. A function of the hydrophobic felt-like mats that are often produced is not obvious but may be prevention of water loss from the substrate. Certain aerial hyphae, however, may differentiate into spore-bearing stalks that at their apices form sporangia containing sporangiospores (many zygomycetes) or sterigmata that bud off conidiospores (many ascomycetes). Alternatively, aerial hyphae at their apices may break up into oidiospores.

All these structures serve vegetative reproduction. For sexual reproduction, many fungi form multihyphal fruit bodies within which meiosis occurs and meiospores are formed. As asexual spores, these can also be dispersed through the air or otherwise disseminated. Particularly in members of the basidiomycetes, the fruit bodies (mushrooms and brackets) can attain large sizes and their morphogenesis has attracted much attention both from a purely scientific and, for the edible species, from a commercial point of view (Wessels, 1993b). These large multicellular structures are not formed by cell divisions within meristems, as in plants, but they are formed by individual hyphae that grow at their tips and seem to "know" how to organize themselves into a distinct multihyphal structure. It is clear that, for the elaboration of these aerial fruit bodies, massive transport of water and assimilates from the assimilative substrate mycelium is required. When the substrate is exhausted, components of the substrate mycelium itself may be broken down and breakdown products reused for the construction of these emergent structures (Wessels and Sietsma, 1979; Wessels, 1993b).

After spores have been dispersed, they must find a substrate in order to germinate and to produce a new mycelium. For saprotrophs this poses no problems, provided the spore lands in an area where dead organic material and enough moisture are available. For biotrophs, however, it is often necessary for a spore to attach to, and to germinate on, the bare surface of the host before infection structures can be formed and the host is penetrated to form an assimilative mycelium. This is particularly clear in parasitic relationships with plants and animals (Cole and Hoch, 1991), but is also evident in the mutually beneficial associations with plants, the mycorrhizas (Harley and Smith, 1983; Read, 1991). In addition, a large number of fungi, an estimated 20% of all species, have evolved as lichens, aerial structures in which the fungus obtains its assimilates from symbiotic algae and cyanobacteria (Honneger, 1993).

This brief overview of fungal biology serves as an introduction to understanding the roles played by hydrophobins. These proteins were discovered while searching for genes expressed during emergent growth in *Schizophyllum commune*. As in many homobasidiomycetes, the primary mycelium that grows from a meiospore forms aerial hyphae but, after mating of two compatible primary mycelia, a secondary mycelium is formed that produces fruit bodies in addition. The cDNAs of a number of abundant mRNAs appearing during emergence of the aerial structures of primary and secondary mycelia of *S. commune* were cloned (Mulder and Wessels, 1986). The most abundantly expressed genes were sequenced, revealing that at least four of the ten cloned genes encoded similar small cysteine-rich hydrophobic proteins (Schuren and Wessels, 1990; Wessels *et al.*, 1995). At that time these proteins were totally unknown. Eventually, the product of one of these genes (*SC3*) was found in the walls of aerial hyphae, while the abundant product of another (*SC4*) was found in walls of hyphae that make up fruit bodies (Wessels *et al.*, 1991a,b). The proteins were present in these walls as complexes, insoluble in a hot solution of 2% sodium dodecylsulfate (SDS), that

could be dissociated into monomers only by treatments with pure formic acid or trifluoroacetic acid, although monomers of these proteins were present in the medium of still cultures. Because of the abundance of hydrophobic residues and their presence in walls, we dubbed these proteins "hydrophobins", a term used earlier to denote any substance conferring hydrophobicity to a microbial surface (Rosenberg and Kjelleberg, 1986).

Around the same time, Stringer *et al.* (1991) found a gene in *Aspergillus nidulans* with homology to the *S. commune* hydrophobin genes. Disruption of this gene caused a phenotype of wettable conidiospores from which the so-called rodlet layer was missing. This indicated hydrophobins as an essential component of hydrophobic rodlet layers, generally observed on fungal spores. We then showed that a single purified hydrophobin from *S. commune* (SC3) could form such a hydrophobic rodlet layer *in vitro* by self-assembly at a water–air interface (Wösten *et al.*, 1993), and that such a layer was formed at the surface of aerial hyphae (Wösten *et al.*, 1994b). It was also found that this hydrophobin could mediate strong attachment of *S. commune* hyphae to solid hydrophobic surfaces (Wösten *et al.*, 1994a). In the meantime, hydrophobin-like proteins were found in all fungi examined (de Vries *et al.*, 1993), while anonymous genes highly expressed in fungi during a variety of developmental processes turned out to encode proteins with clear homology to the *S. commune* hydrophobins (Fig. 1).

2. IDENTITY OF HYDROPHOBINS

It is noteworthy that most of the hydrophobins listed in Fig. 1 were found by sequencing cDNAs representing mRNAs abundantly expressed during certain stages of fungal development without knowing anything about the encoded proteins. Only ABH1, CoH1, cerato-ulmin and cryparin were first identified as proteins, and their genes then cloned by polymerase chain reaction (PCR) using degenerate primers based on determined N-terminal amino-acid sequences. In retrospect, the late discovery of these abundantly occurring proteins is understandable because many occur as SDS-insoluble complexes that can be dissociated into monomers only by using concentrated formic acid or trifluoroacetic acid (de Vries *et al.*, 1993), agents not in common use for protein extraction. In principle, these proteins could have been seen when examining proteins present in media from standing cultures, but only after handling such media with special care because the hydrophobins easily aggregate upon exposure to air forming insoluble complexes. Precisely for this reason, cerato-ulmin (CU) and cryparin (CRYP) were detected early because, on shaking, these Class II hydrophobins formed a milky turbidity that could be dissolved in SDS. Yet, the fact that hydrophobin sequences are so readily found in screens for developmentally regulated sequences indicates that they are derived from the most abundantly expressed fungal genes. Indeed, the

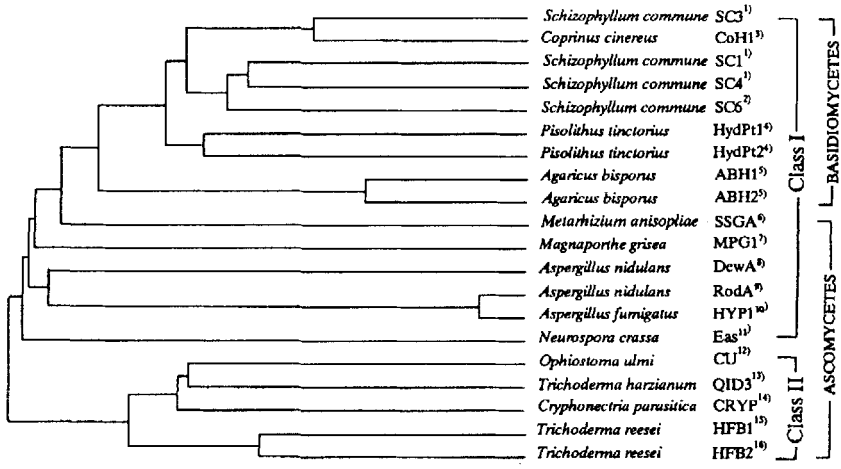

Figure 1 Dendrogram of similarities between aligned hydrophobins obtained by the CLUSTAL programme of the PC/GENE programs package, version 6.60 (Higgins and Sharp, 1988). Numbers in superscript indicate references where sequence information was published or refer to unpublished data. [1]Schuren and Wessels (1990); Wessels *et al.* (1991a). [2]Wessels *et al.* (1995). [3]S.A. Ásgeirsdóttir and L.A. Casselton (unpublished). [4]Martin *et al.* (1995); Tagu *et al.* (1996). [5]Lugones *et al.* (1996); de Groot *et al.* (1996). [6]St Leger *et al.* (1992). [7]Talbot *et al.* (1993). [8]Stringer and Timberlake (1995). [9]Stringer *et al.* (1991); J. Rhodes and W.E. Timberlake, cited in Stringer and Timberlake (1995). [10]Parta *et al.* (1994); Thau *et al.* (1994). [11]Bell-Pedersen *et al.* (1992); Lauter *et al.* (1992); Templeton *et al.* (1995). [12]Yaguchi *et al.* (1993); Bowden *et al.* (1993); Stringer and Timberlake (1993). [13]Lora *et al.* (1994). [14]Zhang *et al.* (1994); Carpenter *et al.* (1992). [15]Nakari-Setälä *et al.* (1996). [16]T. Nakari-Setälä and M. Penttilä (unpublished).

Amino-acid sequences at the N-terminal end located before the first cysteine residue were omitted in the comparison since these include the signal sequence for secretion and in only eight cases (SC3, SC4, CoH1, ABH1, RodA, Eas, CU and CRYP) is the N-terminus of the mature protein known. However, aligning the whole protein sequence, including the signal sequence, results qualitatively in the same type of dendrogram, only the distances become larger. The overall identity of all sequences is only 4.3%, the overall similarity 1.7%.

SC3 and *SC4* genes of *S. commune* were shown to produce 1% and 3.5%, respectively, of the mRNA mass at the time of emergent growth (Mulder and Wessels, 1986), while the record is probably set by the mRNA for cryparin that amounted to 25% of the mRNA mass (Zhang *et al.*, 1994).

The sequence diversity of hydrophobins (Fig. 1) means that isolation of hydrophobin genes on the basis of sequence homology is mostly impossible. For instance, the four hydrophobin genes cloned from *S. commune* do not cross-hybridize (Mulder and Wessels, 1986), even under non-stringent conditions. Only in the case of related species has nucleic acid homology been used to isolate a hydrophobin gene that fulfils a similar function: the hydrophobin gene that is responsible for formation of rodlets on conidia of *Aspergillus fumigatus* was isolated on the basis of its homology to the *RodA* gene of *A. nidulans* (Parta *et al.*,

1994; Thau *et al.*, 1994). This state of affairs means that it is generally unknown how many hydrophobin genes exist in a given fungal species, but the identification of multiple genes in species, such as *S. commune*, *P. tinctorius* and *A. nidulans*, just by screening cDNA libraries, indicates that, in most studied species, only the most abundantly expressed hydrophobin genes may have been identified.

Of the (putative) hydrophobins listed in Fig. 1, only SC3 (Wösten *et al.*, 1993) and SC4 (this laboratory, unpublished data) from *S. commune*, ABH1 from *Agaricus bisporus* (Lugones *et al.*, 1996), CoH1 from *Coprinus cinereus* (S.A. Ásgeirsdóttir and L.A. Casselton, unpublished data), CU from *Ophiostoma ulmi* (Takai and Richards, 1978; Russo *et al.*, 1982) and CRYP from *Cryphonectria parasitica* (Carpenter *et al.*, 1992) have been physically isolated and their properties studied. Wessels (1992) noted that the remarkable property of interfacial self-assembly exhibited by the SC3 hydrophobin of *S. commune* (see below) was earlier observed with CU (Takai and Richards, 1978; Russo *et al.*, 1982) and CRYP (Carpenter *et al.*, 1992). When the amino-acid sequence of CU became available (Yaguchi *et al.*, 1993), Stringer and Timberlake (1993) noted the sequence homology to known hydrophobins. However, whereas interfacial self-assembly of, for instance, SC3, SC4 and ABH1 hydrophobins results in aggregates that are highly insoluble in water, organic solvents and 2% SDS, the aggregates formed by CU and CRYP were found to be unstable in water, and soluble in aqueous ethanol and 2% SDS. In addition, they display a hydropathy pattern that is clearly different from that of hydrophobins like SC3 (Fig. 2). Therefore, Wessels (1994) proposed a distinction between Class I hydrophobins that form highly insoluble assemblages and Class II hydrophobins that form less stable assemblages (e.g. soluble in 60% ethanol or 2% SDS), a distinction supported by the alignment dendrogram shown in Fig. 1. In the Class I hydrophobins, the cysteine doublets are followed by a stretch of hydrophilic amino acids whereas, in Class II hydrophobins, hydrophobic residues immediately follow the cysteine doublets (Fig. 2). Also, fewer amino acids separate the third and fourth cysteine residue in Class II hydrophobins than in Class I hydrophobins. Whether this grouping is correct can only be decided after isolation and characterization of all the listed hydrophobins. However, because most of the hydrophobins tabulated in Fig. 1 have not yet been physically isolated, they can be only tentatively grouped as Class I and Class II hydrophobins on the basis of similarities in hydropathy patterns and solubility characteristics of assemblages. It would not be surprising if some of these hydrophobins exhibit solubility characteristics intermediate between the two classes now distinguished.

On the basis of the available information on hydrophobins, they would seem to have the following characteristics:

1. Hydrophobins are small proteins (100 ± 25 amino acids) that are moderately hydrophobic. The hydrophobicity indices (Kyte and Doolittle, 1982) for mature proteins vary from 0.01 (RodA) to 0.60 (SC3). The overall hydrophobicity thus varies widely.

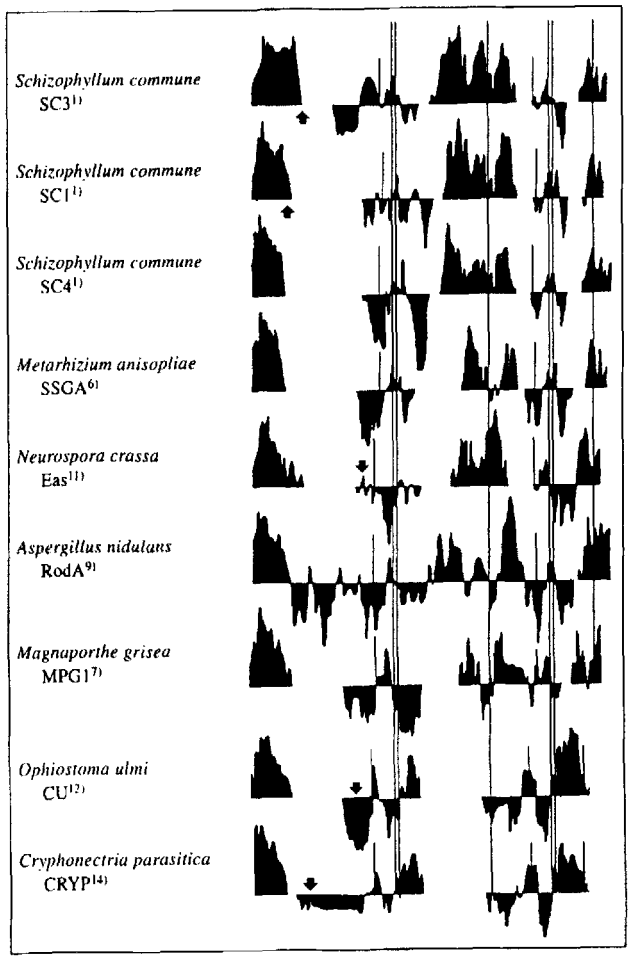

Figure 2 Comparison of hydropathy patterns of selected hydrophobins (SC3, SC1, SC4, SSGA, Eas, RodA, MPG1, CU and CRYP) (for references, see Fig. 1). The patterns were determined using the parameters of Kyte and Doolittle (1982). A six amino-acid window was used and plotted against position in the deduced amino-acid sequence. The hydropathy patterns were then aligned around the first and second cysteine doublet, and around the fourth and eighth cysteine residue leaving gaps in the sequences where the hydrophobic regions (above the lines) alternate with hydrophilic regions. The hydrophobic amino-terminal sequences serve as signal sequences for secretion. The amino termini for the mature hydrophobins, when known, are indicated by arrows. Note that the first seven hydrophobins (Class I) have similar hydropathy patterns, which deviate from those of the last two hydrophobins (Class II). (Modified from Wessels, 1994, with permission from the publisher.)

2. Hydrophobins are all secreted as suggested by the presence of signal sequences. This was actually shown for those hydrophobins in which the amino terminus of the mature protein was determined (arrows in Fig. 2).
3. Hydrophobins have a conserved spacing of eight cysteine residues:

   X_{2-38}–C–X_{5-9}–C–C–X_{11-39}–C–X_{8-23}–C–X_{5-9}–C–C–X_{6-18}–C–X_{2-13}

   in which X signifies any other amino acid, except for tryptophan, which has been reported only in HydPt1, while methionine has been found only in HydPt1, HydPt2, Eas, SSGA and MPG1. Asparagine mostly follows the first cysteine doublet. Of course, the numbers of amino acids that separate the cysteine residues may change as more hydrophobins are sequenced but the recurrent hydropathy patterns around the sequence C–X_{5-9}–C–C in the amino-terminal and carboxy-terminal halves of the molecule are remarkable (Fig. 2). (Note that in the putative QID3 protein listed in Fig. 1, serine substitutes for the second cysteine residue.)
4. Hydrophobins have poor amino-acid homology. For instance, the SC1, SC3 and SC4 hydrophobins, all produced by *S. commune*, are only 39% identical. However, many of the differences concern conservative substitutions so that the similarity between these hydrophobins becomes 80%. If the RodA hydrophobin of *A. nidulans* and the Eas hydrophobin of *N. crassa* are also taken into account, the identity between the five hydrophobins drops to 11% and the similarity to 34%. The similarities between the hydrophobins therefore become most clear when both the conserved spacings of cysteine residues and the hydropathy patterns are compared (Fig. 2).
5. Hydrophobins have the capacity to assemble into an amphipathic protein film when confronted with a hydrophilic–hydrophobic interface, such as between water and air. As indicated above for the Class I hydrophobins, this was shown only for the hydrophobins SC3, SC4, CoH1 and ABH1. However, the hydrophobins Eas (Templeton *et al.*, 1995), MPG1 (Talbot *et al.*, 1993), RodA (Stringer *et al.*, 1991) and HYP1 (Parta *et al.*, 1994; Thau *et al.*, 1994) have all been shown to be part of, or constitute, the hot SDS-insoluble hydrophobic rodlet layer on conidiospores and thus most probably had gone through the interfacial self-assembly process. For the putative Class II hydrophobins, interfacial self-assembly has clearly been established for CU (Takai and Richards, 1978; Russo *et al.*, 1982; Richards, 1993), CRYP (Carpenter *et al.*, 1992) and HFB1 (Nakari-Setälä *et al.*, 1996).
6. As far as is known, all hydrophobins are present as assemblages on the surfaces of emergent hyphal structures.

These criteria delimit the hydrophobins from other cysteine-rich proteins of fungal or other origins. It has been suggested (St Leger *et al.*, 1992; Templeton *et al.*, 1994) that hydrophobins may be related to proteins exhibiting the so-called toxin-agglutinin fold (Drenth *et al.*, 1980; Andersen *et al.*, 1993). For the agglutinins belonging to the chitin-binding family (Raikhel *et al.*, 1993) disulphide

bridges occur between C1–C4, C2–C5, C3–C6 and C7–C8 within the 30–43 amino-acid motifs containing eight cysteine residues. For Class I hydrophobin SC3, it was found that no free SH groups were present in either the monomeric or assembled form, and thus that all cysteines were probably involved in disulphide bridges (de Vries *et al.*, 1993). For the Class II hydrophobin CU, Yaguchi *et al.* (1993) determined that disulphide bridges occur between C1–C2, C3–C4, C5–C6 and C7–C8, emphasizing the recurrent motif in hydrophobins already suggested by the spacing of cysteine residues and hydropathy pattern (Fig. 2), and pointing to two-domain proteins.

If this is the pattern of disulphide bridges found in all hydrophobins, then there is a clear difference in structure between hydrophobins and other high-cysteine proteins. Nevertheless, a comparison between agglutinins and hydrophobins is interesting since lectin activity has been demonstrated for cryparin (Carpenter *et al.*, 1992). In view of the presence of assembled hydrophobins on the surface of walls that mainly consist of polysaccharides (Wessels and Sietsma, 1981), it would not be surprising if a lectin-like binding were involved in anchoring hydrophobins to the wall.

Small extracellular proteins often contain 7–8 cysteine residues. Notable examples are the snake toxins and chitin-binding lectins (Drenth *et al.*, 1980) referred to above, the thionins (Bohlmann and Apel, 1991), the extracellular lipid transfer proteins (Sterk *et al.*, 1991), the so-called defensins (Terras *et al.*, 1995) and the hydrophobic parts of bimodular proteins (Castonguay *et al.*, 1994), all from plants. None of these have the specific spacings of cysteine residues as noted for the hydrophobins nor have they been shown to be active in self-assembly. For the same reasons, there is no compelling evidence at the moment to associate the hydrophobins with other secreted fungal polypeptides, such as toxins (Wnendt *et al.*, 1994) or peptide elicitors (Nespoulous *et al.*, 1992; van den Ackerveken *et al.*, 1993; Rohe *et al.*, 1995) as was proposed by Templeton *et al.* (1994) and Sticklen and Bolyard (1994). However, it should be mentioned that the NIP1 elicitor of *Rhynchosporium secalis* has the first eight of its ten cysteine residues exactly spaced as in hydrophobins (Rohe *et al.*, 1995), emphasizing the possibility that hydrophobins or derivatives of hydrophobins may act as elicitors of the plant defence reaction (Wessels, 1994).

There is an imminent need for probing the three-dimensional structure of hydrophobins before and after interfacial assembly. Whereas the assembly of Class II hydrophobins appears reversible, the assembly of the known Class I hydrophobins must be accompanied by a very stable conformational change, since it cannot be reversed except by using solvents like formic acid and trifluoroacetic acid (TFA). It should also be noted that these proteins are very robust and functionally unaffected by treatments with these aggressive reagents, since their dissociation and assembly can be repeated many times (Wösten *et al.*, 1993). It is only through structural analyses that we can begin to understand the interfacial assembly of these proteins into an amphipathic film with a rough hydrophobic surface of rodlets, and

their remarkable property to attach to surfaces and to reverse their wettability (see below).

3. RODLETS

The structures of concern here are 5–10 nm thick rodlets of varying length organized in bundles or fascicles, in which individual rodlets are laid down in parallel fashion within a single fascicle (Fig. 3). They were first seen by freeze–etching of conidiospores of ascomycetes (Sassen *et al.*, 1967; Hess *et al.*, 1968). They were also detected on hyphae and spores of zygomycetes (Cole *et al.*, 1979; Hobot and Gull, 1981) and basidiomycetes (Bronchart and Demoulin, 1971; Wessels *et al.*, 1972; Gerin *et al.*, 1994). Honneger (1991) found similar rodlets lining the air spaces within thalli of many lichens.

Wessels *et al.* (1972) found rodlets on hyphae of *Schizophyllum commune* decorating the outside of the outer wall layer of (1-3)-α-glucan (S-glucan). Because this glucan was the only component in the untreated wall that showed crystallinity by X-ray diffraction, they made the erroneous suggestion that the rodlets were an aspect of the crystalline S-glucan. Hashimoto *et al.* (1976) detached the rodlet layer from microconidia of *Trichophyton mentagrophytes*, a dermatophyte, and they were the first to publish a careful chemical analysis. The rodlet layer was found to be remarkably resistant to dissolution by most common organic solvents, cell-wall

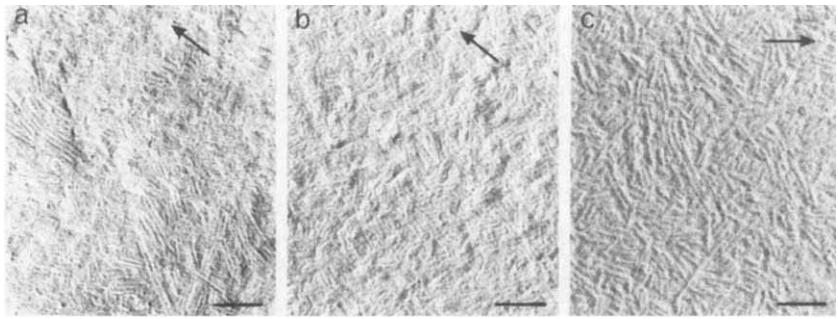

Figure 3 Morphological appearance of assembled SC3 hydrophobin. (a) Freeze–fracturing and shadowing shows the typical rodlets of assembled SC3 at the surface of aerial hyphae. (b) and (c) Similar rodlets are observed after drying down solutions of pure SC3 and shadowing the preparations; at 3.5 μg cm^{-1} the rodlets are shorter (b) than at 0.35 μg cm^{-1} (c). Bar represents 100 nm. Arrows indicate direction of shadowing. (From Wösten *et al.*, 1994b, with permission from the publisher.)

lytic enzymes, detergents, mild acids and alkali treatments, but was solubilized in boiling 1 M NaOH. Protein (80–85%) and glucomannan (7–10%) were found to be the major components of the rodlet layer, in spite of its resistance to degradation with proteases such as trypsin and pepsin. Beever *et al.* (1979), analysing the chemical nature of the rodlet layer of *Neurospora crassa* macroconidia, also noted that this layer was extremely insoluble, and likewise found mainly protein (91%) and a small amount of carbohydrate and lipid. They regarded the small amount of lipid and the rough surface of the rodlet layer responsible for the non-wettability of the surface of the conidia. Similar properties were found for the rodlet layer on conidia of *Aspergillus niger* (Cole *et al.*, 1979) and *Aspergillus nidulans* (Claverie-Martin *et al.*, 1986), while Hobot and Gull (1981) appear to be the first to show a protein subunit as part of the rodlet layer. Working with the zygomycete, *Syncephalastrum racemosum*, they found that alkali extraction removed the rodlet layer from the spores and that a 70% ethanol supernatant of such an extract contained a glycoprotein that ran at approximately 12 kDa on SDS-polyacrylamide gel electrophoresis (SDS-PAGE). By using the method of dissociation of assembled hydrophobin with TFA (de Vries *et al.*, 1993), Templeton *et al.* (1995) have recently shown that the rodlet layer isolated from *N. crassa* conidia is predominantly composed of one protein, the product of the hydrophobin gene *eas*. Similarly, Bidochka *et al.* (1995a) found a small protein as constituent of the rodlet layer of spores of the entomopathogenic *Beauveria bassiana*. N-terminal sequencing of the protein showed the typical C–X_n–C–C–N motif present in hydrophobins.

Freeze–etching has revealed similar rodlet layers on the spores of aerobic bacteria (Holt and Leadbetter, 1969; Aronson and Fitz-James, 1976) and very distinctly on aerial hyphae and spores of *Streptomyces* species (Wildermuth *et al.*, 1971; Williams *et al.*, 1972). It would be extremely interesting to know whether these structures, which probably fulfil the same functions as the rodlet layers of fungi, are composed of hydrophobins or if these rodlets are built of a different material and represent an example of convergent evolution. Unfortunately, very little is know about the chemistry of these prokaryotic rodlet layers. Smucker and Pfister (1978) examined the chemistry of rodlets in the actinomycete *Streptomyces coelicolor* and suggested that they were composed of a polysaccharide complex most similar to chitin. However, the rodlets on the spore coats of aerobic eubacteria (e.g. *Bacillus cereus*) were considered to consist of cysteine-rich protein that resisted SDS extraction (Aronson and Fitz-James, 1976).

As mentioned in the Introduction, a strong indication for involvement of hydrophobins in the formation of the rodlet layer on fungal spores came from experiments in which anonymous genes, isolated on the basis of high expression during conidiogenesis, were inactivated by targeted mutations. This was first shown for *RodA* in *Aspergillus nidulans* (Stringer *et al.*, 1991) and subsequently for *eas* in *Neurospora crassa* (Bell-Pedersen *et al.*, 1992; Lauter *et al.*, 1992). In these cases the targeted mutation not only removed the rodlet layer from the spores but also caused the easily wettable phenotype preventing the spores from being

dispersed through the air. The known allele of *N. crassa* called *eas* (*eas*ily wettable) with the same phenotype (Selitrennikoff, 1976; Beever and Dempsey, 1978) could be complemented by the isolated genes *ccg-2* (Bell-Pedersen *et al.*, 1992) and *bli-7* (Lauter *et al.*, 1992), both allelic to the *eas* gene. The homologue of *RodA* in the human pathogen *Aspergillus fumigatus* was isolated and found to complement the targeted mutation *rodletless* in *A. nidulans* (Parta *et al.*, 1994) whereas a targeted mutation in *A. fumigatus* itself showed the same phenotype as rodletness in *A. nidulans* (Thau *et al.*, 1994). Significantly, the spores of this mutant were as pathogenic for mice as those from the wild-type strain.

The aforementioned genetic experiments only show that hydrophobins are necessary for the formation of rodlets and probably constitute at least one component of the rodlet layer. It has now been shown, however, that single hydrophobins can form rodlet layers *in vitro*. Purified monomers of SC3 hydrophobin of *Schizophyllum commune* in aqueous solution immediately assembled around air bubbles or oil droplets coating these with an SDS-insoluble film (Wösten *et al.*, 1993, 1994a). Upon freeze–fracturing, the side facing the hydrophobic phase showed rodlets that could not be distinguished from those seen on aerial hyphae. Simply drying down an aqueous solution of SC3 on a Formvar grid for electron microscopy, exposing the hydrophobin to a water–air interface, also revealed the rodlet layer after shadowing (Fig 3; Wösten *et al.*, 1993, 1994b). Significantly, the hydrophobicity of the air-exposed surface of the SC3 film was as high (water contact angles of about 110°) as that of the surface of aerial hyphae. This process of interfacial self-assembly of a single hydrophobin into a hydrophobic rodlet layer provides for a remarkably simple mechanism by which hyphae and spores obtain a hydrophobic layer at their surface because it is at this surface that the secreted hydrophobin monomers reach the water–air interface and assemble into an amphipathic film (Wösten *et al.*, 1994b).

Apart from SC3, the SC4 hydrophobin of *S. commune* also forms a hydrophobic rodlet layer *in vitro* (this laboratory, unpublished data), as does the ABH1 hydrophobin of *A. bisporus* (Lugones *et al.*, 1996). CoH1 also shows interfacial self-assembly (S.A. Ásgeirsdóttir, unpublished data) but, as far as is known, none of the other Class I hydrophobins listed in Fig. 1 has been tested for self-assembly and formation of rodlets *in vitro*. Disruption of *RodA* in *A. nidulans* caused complete absence of rodlets on conidiospores and an easily wettable phenotype resulted (Stringer *et al.*, 1991). Yet, a more recent study (Stringer and Timberlake, 1995) showed that such conidia still contain a hydrophobin (DewA) on their surfaces. Apparently DewA alone is not able to produce rodlets on the spores. Disruption of only *DewA* caused the spores to become wetted by a solution containing both 0.2% SDS and 50 mM ethylenediamine tetraacetic acid (EDTA), while disruption of both *RodA* and *DewA* caused a higher hydrophilicity of the spores than disruption of *RodA* alone. After removing a hydrophobin-containing rodlet layer from conidia of *Beauveria bassiana* (Bidochka *et al.*, 1995a) an SDS-insoluble but formic-acid-extractable protein of low molecular mass

(15.4 kDa after oxidation with performic acid) remained (Bidochka *et al.*, 1995b). Since N-terminal amino-acid sequencing proceeded for 24 amino acids only, it was impossible to know whether or not this protein represented a hydrophobin. If so, it was apparently not present in a rodlet layer.

Although the Class II hydrophobins cerato-ulmin and cryparin are secreted into the culture medium, they also cover aerial structures in *Ophiostoma ulmi* (Takai and Hiratsuka, 1980; Svircev *et al.*, 1988) and *Cryphonectria* (Carpenter *et al.*, 1992), respectively. The occurrence of rodlets on surfaces of these fungi was not reported. Therefore, it is uncertain at the moment whether rodlets are a general aspect of assembled hydrophobins.

Interfacial self-assembly resulting in a rodlet layer at a water–air interface, as evident for the SC3 hydrophobin (Wösten *et al.*, 1993), would only be expected to occur in aerial structures. Indeed, submerged hyphae of *S. commune*, which produce hydrophobins, secrete these into the medium as monomers (Wessels *et al.*, 1991a,b; Wösten *et al.*, 1994b). Also, on submerged produced conidia of *A. nidulans*, rodlets were not detected, although the *RodA* transcript was produced, suggesting diffusion of the hydrophobin into the medium (Stringer *et al.*, 1991). Muñoz *et al.* (1995) found submerged conidiospores of *Trichoderma harzianum* to be hydrophilic but aerial spores were hydrophobic, and only these contained a putative hydrophobin. In contrast, both aerial and submerged conidia of *Beauveria bassiana* contained hydrophobin (Bidochka *et al.*, 1995a). From the paper it is not entirely clear whether rodlets, and thus the assembled form of the hydrophobin, were actually seen on the submerged spores. Rodlet layers could have formed from hydrophobin monomers assembled on air bubbles swirled into the medium during shaking (Wösten *et al.*, 1993).

4. SURFACE ACTIVITIES OF HYDROPHOBINS

4.1. SC3 Hydrophobin

Most of the Class I hydrophobins listed in Fig. 1 are known to be produced only in specific structures or under particular environmental conditions. For instance, transcription of *RodA* (Stringer *et al.*, 1991) and *DewA* (Stringer and Timberlake, 1995) in *Aspergillus nidulans* is probably restricted to phialides that produce the conidiospores. Although species like *A. nidulans*, *A. niger*, *A. oryzae*, *Neurospora crassa* and *Penicillium chrysogenum* do produce hydrophobin-like proteins typical for Class I in the culture medium (de Vries *et al.*, 1993; unpublished data), the amounts found were much less than in *Schizophyllum commune*.

On account of its abundance, the SC3 hydrophobin could be easily purified from the medium of 5-day-old standing or shaking cultures of the monokaryon of *Schizophyllum commune*. Although the fungus harbours at least three other hydrophobin genes (Fig. 1), the latter are only substantially active in the dikaryon (Mulder and Wessels, 1986) unless the monokaryon carries alleles that induce monokaryotic fruiting (Yli-Mattila *et al.*, 1989a; see Table 1). SC3 is the most prominently secreted protein of the monokaryon while the dikaryon mainly secretes SC4 together with varying amounts of SC3 (Wessels *et al.*, 1991a). Irrespective of culture conditions, the genes for these hydrophobins become active after 2–3 days of cultivation (Wessels *et al.*, 1987) and the hydrophobins are secreted into the medium.

In standing cultures, in which emergent growth occurs, the hydrophobins in the medium accumulate largely in a soluble state; in shaken cultures they mostly occur as very fine insoluble particles because of aggregation at the increased medium–air interface (Wessels *et al.*, 1991a,b; Wösten *et al.*, 1993). In both cases the mycelium can be filtered off on nylon cloth leaving the hydrophobins in the filtrate. They are then purified essentially as described by Wösten *et al.* (1993, 1994b). After mixing with air or heating the medium to 100°C, to achieve complete aggregation, the aggregates are spun down, treated with concentrated TFA and, after removing TFA by evaporation, the dissociated hydrophobins dissolved in water. After removing particulates, the solution is made 60% in ethanol leaving the monomeric hydrophobins soluble, but polysaccharides and a 15 kDa contaminating protein are precipitated (Wösten *et al.*, 1993). Further purification is achieved by precipitation of the hydrophobin at higher ethanol concentration, dissolution of the precipitated monomers in water and repeating the procedure of interfacial precipitation and TFA dissociation. Using the standard monokaryon 4–39, about 20 mg of purified SC3 is obtained per litre of medium. The protein can be stored for some time in 60% aqueous ethanol keeping it in monomeric form. For assembly, this solution can be diluted with degassed water to lower the ethanol concentration to less than 5%.

For experiments involving radioactive SC3, $^{35}SO_4^{2-}$ is included in the culture medium. SC3 was shown to be a glycoprotein that stained in a periodic acid–Schiff reaction, bound the lectin conconavalin A, and contained mannose. According to its protein sequence, one would predict a molecular mass of 9830 Da but mass spectroscopy showed 14 200 Da (this laboratory, unpublished data). Assuming no other post-translational attachments, the presence of about 23 mannose residues can be calculated, probably linked to the abundantly occurring serine and threonine residues since no putative *N*-glycosylation site is found in SC3 (Schuren and Wessels, 1990). In SDS-PAGE the SC3 hydrophobin runs slower than expected, namely at a position corresponding to marker proteins of 24 kDa; after oxidation with performic acid, disrupting disulphide bonds by oxidizing cystine to cysteic acid, it migrates at a position corresponding to 28 kDa.

When bubbling air or nitrogen gas through an aqueous solution of purified SC3, or simply by shaking or vortexing the solution, a milky suspension is obtained that

shows irregularly shaped gas vesicles in the light microscope (Wösten *et al.*, 1993). Upon standing, these vesicles float to the surface; by applying a vacuum they collapse, leaving aggregated SC3 that can be easily centrifuged down and which is insoluble in 2% SDS at 100°C. This suggested that the gas vesicles are coated with a highly stable film of assembled SC3. Apparently this is accompanied by a considerable lowering of the surface tension of the water, since the gas vesicles attain various odd shapes. Films of about 10 nm thickness could be visualized by sectioning the precipitate obtained by bursting coated air vesicles, while freeze–fracturing and direct surface shadowing revealed the typical pattern of rodlets seen on the surface of aerial hyphae and discussed in the previous section. SC3, which had been assembled by drying down an aqueous solution on a hydrophilic glass surface (water contact angle 15°) produced a hydrophobic surface corresponding to water contact angles up to 95°. Solutions dried on the surface of the thin mutant of *S. commune*, a mutant not producing SC3 nor aerial hyphae, and having a hydrophilic surface, produced a surface hydrophobicity of 110°, close to the value of 115° found for the surface of the wild-type monokaryon (Wösten *et al.*, 1993). The presence of a water–air interface apparently leads to a stable conformational change that links the SC3 molecules tightly to each other in an amphipathic two-dimensional film, which exposes the typical fascicles of rodlets at its hydrophobic side. Indeed, if only protein and carbohydrate are present and no lipids, then this conformational change must result in an orientation of polar and apolar amino acids to different sides of the film, the mannose residues probably being exposed at the hydrophilic side.

Because gasses have a hydrophobic character, the ability of an oil suspension in water to provide a suitable interface for SC3 assembly was investigated. Indeed SC3 stabilized oil droplets in water by coating these with a 10 nm thick SDS-insoluble protein film (Wösten *et al.*, 1994a). Again, the shape of the oil droplets indicated a considerable drop in the surface tension. Freeze–etching and shadowing revealed that the amphipathic film exhibited rodlets on its hydrophobic side but had a smooth appearance at its hydrophilic side. Immersing a hydrophobic sheet of plastic like Teflon into an aqueous solution of SC3 caused SC3 to assemble on the hydrophobic surface. After removal of the Teflon sheet from the solution, water flowed evenly over its surface, indicating that it had become hydrophilic. After drying it was found that water contact angles (θ) on the surface had decreased from 108° to 48° and that 5.9×10^{12} molecules SC3/cm^2 had adsorbed. Treatment with 2% SDS for 10 min at 100°C, removed only 13.5% of the adsorbed hydrophobin, while water contact angles rose slightly to 62°. This contrasts with other proteins, such as bovine serum albumin, which also adsorb to plastics but are completely removed by hot 2% SDS.

Adsorption of SC3 could occur from very dilute solutions. At 2 μg ml^{-1}, saturation was reached after 16 h but at 20 μg ml^{-1}, saturation of the surface was reached after only 2 min of incubation. It was concluded that the exposure of SC3 to the hydrophilic–hydrophobic interface induces a conformational change in the

hydrophobin monomers leading to their assembly into a stable amphipathic film strongly attached with its apolar groups to the hydrophobic plastic and exposing polar groups at the surface (Wösten *et al.*, 1994a).

Materials with lower surface hydrophobicities than Teflon, when immersed in an aqueous SC3 solution, caused fewer molecules of SC3 to assemble at their surfaces (Wösten *et al.*, 1994a). To investigate whether the degree of hydrophobicity was the only factor determining assembly, a continuous hydrophobicity gradient surface, displaying water contact angles ranging from 20° up to 107°, was made by coating glass with dichlorodimethylsilane according to Elwing *et al.* (1987). The amount of assembled SC3, defined as SC3 on the surface becoming insoluble in hot SDS after immersing the gradients for 16 h in SC3 (2 μg ml^{-1}), sharply increased in the region of the gradient surface displaying advancing water contact angles between 60° and 90°, then more slowly to the 107° region, i.e. the hydrophobic end. Here, the absorbed SC3 decreased the advancing water contact angle from 107° on the bare gradient surface to 60° on the protein-coated surface (to 39° before extraction with SDS) (Wösten *et al.*, 1995). It is thus clear that the interfacial tension at the solid–liquid interface (γ_{SL}) is the major factor that induces SC3 assembly.

The surface activity of SC3 was compared with that of other proteins using the method of axisymmetric drop shape analysis by profile (ADSA-P) (van der Vegt *et al.*, 1996). In this method (Rotenberg *et al.*, 1983; Noordmans and Busscher, 1991), a drop (100 μl) of water or buffer in which the protein is dissolved is placed on the surface of fluoroethylenepropylene (FEP-Teflon), and changes with time with the shape and contact angle of the axisymmetric droplet are recorded, allowing calculations of the interfacial tension changes to the hydrophobic solid (γ_{SL}) and to the water–air (vapour) interface (γ_{LV}). SC3 at 100 μg ml^{-1} buffer (10 mM KPi, pH7) caused a large drop in the liquid surface tension (γ_{LV}) from 72 to 43 mJ m^{-2} and even to 32 mJ m^{-2}, when the hydrophobin was dissolved in water. At this low concentration of protein, the drop in surface tensions caused by other proteins examined (bovine serum albumin, human immunoglobin G, chicken egg white lysozyme, bovine pancreatic ribonuclease A, bovine milk α-lactalbumin) was much less, reaching a minimum of 54 mJ m^{-2} with lysozyme.

SC3 thus proves to be a powerful surface-active protein, particularly when dissolved in pure water. The kinetics of change in surface activity suggests that the large drop in γ_{LV} is mainly caused by a conformational change in the protein. Surprisingly, the surface tension at the solid–liquid interface (γ_{SL}) did not decrease, as expected from the measurements of Wösten *et al.* (1994a, 1995), and observed for all other proteins tested, but slightly increased from about 42 to 43 mJ m^{-2} with fluid contact angles increasing from 111° to 122°. Adsorption of SC3 to the Teflon thus made the surface more hydrophobic and not hydrophilic as expected. This was explained by assuming that, in this case, the conformational change caused by adsorption of an SC3 layer to the Teflon leads to adsorption of a second layer that exposed its hydrophobic side to the aqueous solution. This second layer would be

sheared from the bottom layer when removing the Teflon sheet from the aqueous solution so that, after drying of the Teflon, a hydrophilic surface remains as detected by water contact angles (van der Vegt *et al.*, 1996).

The atomic composition of microbial surfaces can be analysed by X-ray photoelectron spectroscopy (XPS) (Rouxhet *et al.*, 1994). XPS is based on irradiating a surface with X-rays and analysing the kinetic energy of the photo-ejected electrons. This provides an elemental surface analysis with an analysed depth in the nanometre range. Since the photoelectron kinetic energy depends on the chemical state of the element, different functional groups can also be distinguished. Between the third and sixth day, water contact angles measured at the surface of an *S. commune* monokaryon rose from 30° to 125° and XPS showed this to be accompanied by a rise in the N/C ratio from 0.08 to 0.15, and a rise in the S/C ratio from 0.002 to 0.007 (Wösten *et al.*, 1994c). However, these values remained lower than those measured on artificially assembled SC3, possibly owing to absorption of extraneous materials to the hydrophobic hyphae. Measurements on SC3 films assembled *in vitro* (Wösten *et al.*, 1994c) showed an atomic composition at the hydrophobic side (θ 95°) close to that predicted from the known amino-acid composition. Only the O/C ratios were higher than the values calculated from the polypeptide chain but this could be accommodated by assuming the presence of 11 anhydromannose molecules in this glycoprotein. XPS of the SC3 film assembled on polytetrafluoroethylene (PTFE, Teflon), exposing the hydrophilic side (θ 48°), showed N/C, S/C and C=O/C ratios, which were significantly lower than those at the hydrophobic side while the N/S ratios were similar. This would indicate an orientation of peptide bonds and amino-acid chains towards the hydrophobic side and possibly sugar residues oriented towards the hydrophilic side. This emphasizes the amphipathic nature of the SC3 film.

Since experimental XPS values obtained at the hydrophobic side of assembled SC3 were similar to those expected for the whole protein, emitted photoelectrons must have originated from all parts of the 10 nm-thick film. However, few electrons were expected to be emitted after excitation from a depth exceeding 5 nm (Rouxhet *et al.*, 1994). Possibly the serrated hydrophobic surface caused by the presence of rodlets in random orientation allowed for emission of photoelectrons from all parts of the film while the smooth hydrophilic surface would prevent photoelectrons from deeper parts from escaping (Wösten *et al.*, 1994c).

It has been suggested that the topography of the rodlet surface plays a significant role in decreasing its wettability (Fisher *et al.*, 1978; Beever *et al.*, 1979). If no lipids are attached, it is indeed remarkable that the apolar side chains of hydrophobic amino acids in a hydrophobin such as SC3 could produce surfaces showing water contact angles of 95° when assembled on glass (Wösten *et al.*, 1993) or even 122° when assembled in a double layer on Teflon (van der Vegt *et al.*, 1995), while water contact angles on a bare Parafilm or Teflon surface measure 105–108°. Although surface roughness clearly influences surface wettability (Huh and Mason, 1977; Hazlett, 1992), measurements of Busscher *et al.* (1984) have

indicated that water contact angles increase only if the roughness of a surface is caused by structures exceeding 100 nm in size whereas the hydrophobin rodlets show a periodicity of about 10 nm. Nevertheless, it would be interesting to see whether water contact angles would increase by etching a rodlet pattern on a hydrophobic surface, when compared to a smooth surface of the same material.

4.2. Cerato-ulmin

After Zentmeyer (1942) indicated a toxin of *Ophiostoma (Ceratocystis) ulmi* as responsible for causing Dutch elm disease, Takai (1974) was the first to propose a small protein produced in the culture medium, named cerato-ulmin (CU) (Fig. 1), as the phytotoxin. This was mainly based on a correlation of cerato-ulmin production in shaken cultures and aggressiveness of the isolated strains (Takai, 1974, 1980; Brasier *et al.*, 1990). Although almost half of the amino-acid sequence of cerato-ulmin was already known in 1979 (Stevenson *et al.*, 1979), it was not until 1993 that the complete sequence of the 75 amino acids constituting the protein was reported (Yaguchi *et al.*, 1993). On the basis of this information, Bolyard and Sticklen (1992) assembled a gene that could be expressed in *E. coli* to give approximately 80 μg protein per litre, while Bowden *et al.* (1993) cloned the gene and determined the complete sequence including the signal sequence for secretion (Fig. 2). Surprisingly, disruption of the gene for cerato-ulmin in an aggressive strain of *O. ulmi* had no effect on the pathogenicity of the fungus (Bowden *et al.*, 1996).

The remarkable properties of cerato-ulmin were described by Takai and Richards (1978) and Russo *et al.* (1982), and more recently summarised by Richards (1993). The protein is very surface active and, as the SC3 hydrophobin, assembles around gas bubbles at concentrations in the nanogram per millilitre range. This property was therefore used to isolate and purify the protein from the medium in which it accumulated at up to 25 μg ml^{-1}. Unlike SC3, however, the presumedly amphipathic films that formed were unstable. The milky solution that arose after shaking or bubbling (containing odd-shaped coated air vesicles described as "rods" and "fibrils") turned clear on centrifugation or applying positive pressure. Apparently the assemblages went into solution, although microscopic "units" were still detected and the real solubility of the protein was considered low. Whereas films formed by SC3 are extremely stable to 60% ethanol or 2% SDS, these solvents readily dissolve assembled cerato-ulmin. Cryparin, produced in abundance in the culture medium by the phytopathogen *Cryphonectria parasitica* behaved similarly (Carpenter *et al.*, 1992). Because of their solubility characteristics, these proteins were called Class II hydrophobins (Wessels, 1994), and it is interesting to note that their structure and hydropathy pattern are also somewhat different from that of the Class I hydrophobins (Figs 1 and 2). However, how these differences translate into a causal relationship between protein structure and stability of the assembled films is unknown.

Both cerato-ulmin (Takai and Hiratsuka, 1980; Svircev *et al.*, 1988) and cryparin (Carpenter *et al.*, 1992) occur in abundance on aerial hyphae and sporulating structures of surface cultures. As the Class I hydrophobins, their function may be related to the emergence of these structures. Only colonies of strains of *C. parasitica* (Carpenter *et al.*, 1992) and *O. ulmi* (Takai, 1980) with a high production of Class II hydrophobins appeared fluffy and aerial. Bowden *et al.* (1996) found that disruption of the cerato-ulmin gene caused a phenotype with sparse production of aerial hyphae and a less hydrophobic surface than wild-type. Supposedly, when present at the wall–air interface, the assembled films of Class II hydrophobins would present their hydrophobic side to the air and thus might be washed away less easily than their solubility in water suggests. In addition they might be anchored to the wall polysaccharides by a lectin-like activity as indicated for cryparin (Carpenter *et al.*, 1992). Whether or not a rodlet structure is present at these hydrophobic surfaces seems not to have been investigated.

5. FORMATION OF EMERGENT STRUCTURES

5.1. Formation of Aerial Hyphae

Using *in vitro* translation of mRNAs, Dons *et al.* (1984) found the *SC3* gene (then not known to encode a hydrophobin and called *ID10*) to be perhaps the most abundantly expressed gene at the time of emergence of aerial hyphae in the monokaryon of *Schizophyllum commune*. Subsequently, several observations on the regulation of this gene suggested an involvement in the formation of aerial hyphae.

1. Formation of aerial hyphae by the monokaryon after 2–3 days in surface culture always coincides with a rise in *SC3* mRNA (Mulder and Wessels, 1986) owing to transcriptional activation of the gene (Schuren *et al.*, 1993c).
2. The frequently occurring spontaneous and recessive *thn* mutation, which blocks all emergent growth (i.e. formation of aerial hyphae in the monokaryon and, when homozygous, formation of both aerial hyphae and fruit bodies in the dikaryon) suppresses expression of *SC3*, indicating that *THN* is required for expression of *SC3* (Wessels *et al.*, 1991b).
3. A heterokaryon containing nuclei with different *B*-mating-type genes but common *A*-mating-type genes (a common-*MAT*A heterokaryon), typified by septal dissolution, continuous nuclear migration and few aerial hyphae (Raper, 1966), has a very low expression of *SC3* (Ásgeirsdóttir *et al.*, 1995). It was earlier found that the same phenotype occurs in a homokaryon (a mycelium with only one genetic type of nucleus) carrying a so-called constitutive mutation in the *MAT*B gene (*mat*B^{con}). This mutation also causes down-regulation of *SC3* (Ruiters *et al.*, 1988). Together, these

studies indicate that the *MAT*B genes are involved in regulation of *SC3*. However, assigning a role for SC3 in formation of aerial hyphae was problematic because transcription of the gene was not confined to aerial hyphae but also abundantly occurred in submerged growing hyphae even in shaken cultures in which aerial growth could not occur (Wessels *et al.*, 1987).

The solution to this problem came with the identification of the product of the *SC3* gene as a hydrophobin (Schuren and Wessels, 1990; Wessels *et al.*, 1991a,b). It was then realized that it was not so much cell-type specific gene activation that brought about cell differentiation but rather the deposition of an insoluble form of the hydrophobin in the wall. When it was found that soluble SC3 hydrophobin monomers could assemble at a water–air interface into an SDS-insoluble amphipathic protein film exposing rodlets at its hydrophobic side (Wösten *et al.*, 1993, 1994b), a mechanism as shown in Fig. 4 became obvious. That SC3 is instrumental in the generation of hydrophobic aerial hyphae was proved by disrupting the *SC3* gene (Wösten *et al.*, 1994a). The mutant monokaryon, now unable to form SC3 hydrophobin, made few aerial hyphae compared to wild-type, at least in Petri dishes that were tightly sealed. In non-sealed Petri dishes, aerial hyphae did form but these had a hydrophilic surface (van Wetter *et al.*, 1996).

The scheme given in Fig. 4 assumes that, in a hypha that breaches the interface of culture medium and air, the hydrophobin monomers can no longer freely diffuse into the medium but remain in the wall. Those reaching the wall–air interface assemble into an insoluble SC3 monolayer coating the hypha and conferring hydrophobicity to the surface. Here the assembled hydrophobin could be immunolocalized (Wösten *et al.*, 1994b). Whether there are special mechanisms that direct hyphal tips to grow towards the air and cause them to breach the surface is unknown. Anyway, once this happens the hyphal surface, including the tip, is probably quickly covered with assembled SC3 and the hypha is irreversibly determined for growth into the air because of the hydrophobicity of its surface. Note that at the growing apex expansion of the SC3 assemblage could occur by continuous intercalation of SC3 monomers into the SC3 film.

Implicit in the above scheme is that hydrophobins are secreted at hyphal tips. A correlation between apical wall growth and protein secretion was shown by Wösten *et al.* (1991) and more specifically the secretion of SC3 hydrophobin was shown to occur at growing hyphal tips (Wösten *et al.*, 1994b). Recently, we showed that apical wall growth does indeed occur in hyphae emerging into the air (this laboratory, unpublished data). In order to assemble at the outer surface, the hydrophobin monomers have to be translocated over the wall. Even in the absence of a steep diffusion gradient in these aerial hyphae, as in hyphae growing submerged, this could be achieved by a mechanism proposed by Wessels (1990, 1994). According to this "bulk-flow" mechanism, proteins are not so much diffusing through pores in the wall but flow from the inside to the outside of the wall together with visco-elastic polysaccharides before these become cross-linked

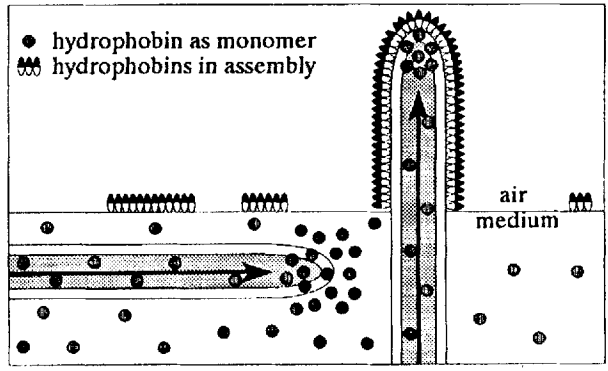

Figure 4 Proposed model for the formation of the hydrophobic rodlet monolayer at the surface of aerial hyphae. Hydrophobin monomers are indicated as grey spheres and hydrophobins in self-assembled films as oval structures with black (hydrophobic) and white (hydrophilic) halves to indicate the conformational change they have undergone. The arrows within the hyphae symbolize transport of newly synthesized SC3 hydrophobin monomers to the growing tip, probably via exocytotic vesicles, where the hydrophobin is secreted. In submerged hyphae, the hydrophobin monomers diffuse into the medium. In aerial hyphae the secreted monomers self-assemble when they reach the interface between the hydrophilic wall and hydrophobic air. Here they form an insoluble amphipathic monolayer with the hydrophobic rodlet-decorated side facing the air. SC3 hydrophobin monomers present in the medium may assemble at the surface of the medium or on hydrophobic walls of the culture vessel. (From Wösten *et al.*, 1994b, with permission from the publisher.)

and crystalline to form the rigid wall. By this mechanism the contents of vesicles fusing with the plasma membrane at the very apex would be completely carried to the surface of the wall. However, it is improbable that the amount of SC3 thus secreted would precisely equal the need to cover the hyphal surface with a monolayer of assembled SC3. Wösten *et al.* (1994b) showed that drying an SC3 solution on a glass slide caused the generation of a monolayer of assembled SC3 at the water–air interface but, because this layer at the same time destroyed the interface, a variable amount of SC3 monomers – depending on their concentration – remained between the glass and the film. One would therefore expect the presence of monomers of SC3 in the wall, in addition to the assembled SC3 at the surface. However, only the latter could be immunolocalized (Wösten *et al.*, 1994b). Possibly the wall fabric prevented the antibodies from reaching their target.

It is interesting to note that the SC3 hydrophobin of *Schizophyllum commune* is related more closely to the CoH1 hydrophobin of *Coprinus cinereus* than to the other hydrophobins (SC1, SC4, SC6) of *S. commune* (Fig. 1). As SC3, CoH1 seems to be required for formation of aerial hyphae; a mutant that does not form aerial hyphae nor aerial oidia does not express *CoH1*. Since the two species are not closely related, this would suggest that the relatedness of SC3 and CoH1 is based on similar

functions, and that the other hydrophobins of *S. commune* are tailored for different purposes.

If the SC3 hydrophobin were needed only for the emergence of aerial hyphae, one would not expect this protein to be secreted in such large quantities by submerged growing hyphae. Of course, it is possible that this is an artefact of the unnatural way of cultivating the fungus in a liquid medium (whether gelled with agar or not). Very few fungi live in water (the water moulds belong to the oomycetes and are not true fungi, see e.g. Cavalier-Smith, 1993). Certainly, wood-rotting species such as *S. commune* do not grow naturally in water but in wood. A hydrophobin like SC3 could attach a *S. commune* hypha tightly to a hydrophobic substrate by assembling at the interface of the hydrophilic wall and the hydrophobic solid (Wösten *et al.*, 1994a). Wood contains hydrophobic components such as lignin. It is therefore possible that production of a hydrophobin like SC3, by assimilating substrate hyphae, serves an important function because it attaches the fungus to its natural substrate. A similar function, apart from conferring hydrophobicity to aerial structures, could be assigned to Class II hydrophobins produced in abundance in the culture media of *Ophiostoma ulmi* and *Cryphonectria parasitica*, both tree pathogens.

5.2. Formation of Fruit Bodies

The large fruit bodies of the homobasidiomycetes (mushrooms, brackets, toadstools) and of some ascomycetes (discomycetes, such as the morels and truffles) are the most spectacular emergent structures elaborated by fungal hyphae. Their morphogenesis has been studied both from a theoretical and a practical point of view (see Wessels, 1993b), the latter because some of them, such as fruit bodies of *Agaricus bisporus*, *Lentinus edodes* and *Pleurotus ostreatus* are an important agricultural product. *Schizophyllum commune*, though commercially worthless, is by far the easiest to fruit in the laboratory and therefore is genetically the best studied homobasidiomycete (Raper, J.R., 1966; Raper, C.A., 1988).

As in most homobasidiomycetes, fruit bodies of *S. commune* are generally formed on a secondary mycelium, that is a heterokaryon arising after mating two primary mycelia originating from spores (homokaryons that have one nucleus per cell and thus are also called monokaryons). The secondary mycelium mostly contains hyphal compartments with two nuclei of opposite mating type lying closely together, and hence it is called a dikaryon. During nuclear division in apical cells, a clamp connection is formed at the septum that divides the two new cells; clamp connections are thus a diagnostic feature for the secondary mycelium. The whole mating process, the maintenance of the dikaryon, and the formation of fruit bodies is controlled by the mating-type genes (Table 1). Only when the two mating mycelia carry different *MAT*A and different *MAT*B genes can sexual morphogenesis proceed. If only the *MAT*B genes are different, nuclei are exchanged, septa are

Table 1 Gene expression and emergent growth in *Schizophyllum commune.*

Genotype	mRNA from *SC3*	mRNA from *SC1, SC4, SC6, SC7, SC14*	Aerial hyphae	Fruit bodies
*MAT*Ax *MAT*Bx	+*	−*	+	−
*MAT*A≠ *MAT*B≠	+	+	+	+
*mat*A^{con} *mat*B^{con}	+	+	+	+
*MAT*A= *MAT*B≠	−	−	−	−
*MAT*Ax *mat*B^{con}	−	−	−	−
*MAT*A≠ *MAT*B=	+	−	+	−
*mat*A^{con} *MAT*Bx	+	−	+	−
*MAT*Ax *MAT*Bx *thn*	−	−	−	−
*MAT*A≠ *MAT*B≠ *thn/thn*	−	−	−	−
*MAT*Ax *MAT*Bx *fbf*	+	−	+	−
*mat*A^{con} *MAT*B^{con} *fbf*	+	−	+	−
*MAT*A≠ *MAT*B≠ *fbf/fbf*	+	−	+	−
*MAT*Ax *MAT*Bx *mfa*	+	+	+	+

* The + sign indicates an abundance of mRNAs ranging from 0.07% (for *SC14*) to 1.0% (*SC3*) and 3.5% (*SC4*) of the total mRNA mass. The − sign indicates an abundance of less than 0.01%, except for *SC4*, where it indicates less than 0.1%.

dissolved and nuclear migration ensues. The resulting heterokaryon has a flat morphology (few aerial hyphae) correlated with repression of the *SC3* hydrophobin gene (Ásgeirsdóttir *et al.*, 1995). If only the *MAT*A genes are different, then primary mycelia fuse – a process independent of the mating-type genes – but nuclei do not migrate. The formation of clamp connections is, however, initiated but not completed; they do not fuse with subterminal cells resulting in so-called pseudo-clamps. None of these semicompatible matings produces fruit bodies; this apparently requires the presence of different *MAT*A and *MAT*B genes.

Although for simplicity the genetic entities governing mating are referred to as *MAT*A and *MAT*B genes, these entities are rather complex genetic loci (for review, see Kües and Casselton, 1992; Casselton and Kües, 1994). What is called *MAT*A is actually a locus containing a series of different alleles that each, in principle, contain two genes, *HD1* and *HD2* (named after the homeodomain sequences they contain). The interaction was shown to be between an *HD1* gene in one nucleus and an *HD2* gene in the other nucleus belonging to the same allelic series. *HD1* and *HD2* genes within the same nucleus do not interact because they belong to different allelic series. Interactions between HD1 and HD2 gene products are thought to produce a gene-activating regulator because of the presence of homeodomains in the proteins. Specific interactions between HD1 and HD2 proteins of *Coprinus cinereus* were recently demonstrated *in vitro* (Banham *et al.*, 1995). It was also known that a rare mutation, indicated as *mat*A^{con} in Table 1,

gives a homokaryotic mycelium a phenotype as if it contains two different *MAT*A genes. This was shown to be due to a deletion effectively fusing an *HD1* and an *HD2* gene within a *MAT*A locus in such a way that the recognition sequence is deleted and a presumed DNA-binding domain (HD) from one gene becomes associated with the activating domain of the other gene (Banham *et al.*, 1995). Although *MAT*B genes appear genetically similar to *MAT*A genes, their interactions seem to involve quite different processes, since genes within these complexes encode multiple pheromones and pheromone receptors (Wendland *et al.*, 1995).

As shown in Table 1, formation of normal fruit bodies in which meiosis occurs requires the presence in a heterokaryon of different *MAT*A and different *MAT*B genes (*MAT*A≠ *MAT*B≠), or the presence in a homokaryon of constitutive mutations in both *MAT*A and *MAT*B (*mat*A^{con} *mat*B^{con}). These genetic conditions allow for the accumulation of dikaryotic transcripts (Mulder and Wessels, 1986; Ruiters *et al.*, 1988). Among these are the mRNAs of the hydrophobin genes *SC1*, *SC4* and *SC6*, as well as the transcripts of two hydrophilic cell-wall proteins *SC7* and *SC14* (Schuren *et al.*, 1993a). By performing run-on experiments on isolated nuclei, evidence was presented that the appearance of these mRNAs is transcriptionally regulated (Schuren *et al.*, 1993c), while in the case of the *SC4* gene, *cis*-regulatory elements responsive to products of the *MAT* genes were indicated (Schuren *et al.*, 1993b). Other regulatory genes have been implicated in the fruiting process (Table 1). Among these are the *THN* gene without which no emergent growth occurs in monokaryon and dikaryon, and none of the hydrophobin genes (*SC3*, *SC1*, *SC4* and *SC6*) are activated (Wessels *et al.*, 1991b). Another gene found necessary for fruit-body formation is *FBF*. The mutant *fbf* was isolated as a frequently occurring mutation that suppresses fruiting in a *mat*A^{con} *mat*B^{con} strain but allows for the formation of abundant aerial hyphae (Springer and Wessels, 1989). At the same time the mutation abolishes the formation of all dikaryon-specific transcripts but allows for high expression of the *SC3* hydrophobin gene. When homozygously present (*fbf/fbf*) in a *MAT*A≠ *MAT*B≠ mycelium, the phenotype of this secondary mycelium is not a dikaryon because incipient clamp connections fail to fuse with subterminal cells, locking one of the nuclei generated by mitosis in the pseudo-clamp. The result is a heterokaryon in which all hyphal compartments, except the apical compartment, are essentially monokaryotic (Springer and Wessels, 1989; Wessels *et al.*, 1995). In this *MAT*A≠ *MAT*B≠ *fbf/fbf* heterokaryon, no transcripts from the *SC1*, *SC4* and *SC6* hydrophobin genes, nor other dikaryon-specific transcripts are produced, but the gene for the SC3 hydrophobin is very active (Springer and Wessels, 1989). Finally, monokaryons may carry so-called haploid fruiting alleles (*hfa*; Table 1). These were interpreted as relaxed versions of genes that normally operate downstream in the regulation cascade from mating-type genes to fruiting genes and therefore allow for fruiting (though not meiosis) in the absence of different mating-type genes (Yli-Mattila *et al.*, 1989a). In these fruiting monokaryons, not only the *SC3* hydrophobin gene but also the *SC1*, *SC4* and *SC6* hydrophobin genes are expressed (Ruiters *et al.*, 1988; Yli-Mattila *et al.*, 1989a).

When grown from a mycelial macerate, hydrophobin genes in the dikaryon are switched on 2–3 days after inoculation, the time at which formation of aerial hyphae and fruit bodies is initiated (Mulder and Wessels, 1986). The levels of hydrophobin mRNAs then rise quickly (*SC3* and *SC4* mRNAs reaching levels of 1% and 3.5% of the total, respectively), but drop sharply when cultures run out of exogenous nitrogen and carbon. It was known that, under such conditions of starvation, growth of the fruit bodies continues, the substrate mycelium is degraded, and massive transport of water and assimilates occurs from the substrate mycelium into the developing fruit bodies (Wessels and Sietsma, 1979; Ruiters and Wessels, 1989; Wessels, 1993b). It was therefore interesting to see that, although the mRNAs from the *SC3*, *SC1*, *SC4* and *SC6* hydrophobin genes sharply decrease in the mycelium as a whole, their concentrations stay very high in the developing fruit bodies, except for the mRNA of the *SC3* hydrophobin gene, which is always expressed at a low level in the developing fruit bodies (Mulder and Wessels, 1986). This indicates that the hydrophobins SC1, SC4 and SC6 play some role in fruit-body formation but that the SC3 hydrophobin may be less important in this process. With a specific antiserum against SC3, it was recently shown that the SC3 hydrophobin is only present on aerial hyphae, including those that cover the fruit bodies, but not on hyphae of the plectenchyma that make up the major part of the fruit-body tissue (Ásgeirsdóttir *et al.*, 1995). Moreover, a dikaryon homozygous for a targeted mutation in the *SC3* gene produces normal sporulating fruit bodies but any aerial hyphae that form are hydrophilic (van Wetter *et al.*, 1996). Apparently these aerial hyphae do not produce SC4 or SC4 cannot functionally substitute for SC3.

Within fruit bodies, differentiation of hyphae occurs, in the sense that the plectenchyma cells produce typical dikaryotic transcripts and proteins. However, the covering aerial hyphae display a more monokaryotic pattern of gene expression, prompting a re-examination of the sites of secretion of these proteins by whole colonies of secondary mycelium (Ásgeirsdóttir *et al.*, 1995). It was found that the SC3 hydrophobin is secreted in another region of the colony than the dikaryon-specific proteins, including the SC4 hydrophobin, which are typically formed in areas supporting fruit bodies. It was found that in nascent aerial hyphae expressing *SC3*, the two nuclei containing different *MAT*A and *MAT*B genes are present at some distance from each other. Since in a common-*MAT*A heterokaryon (with interaction between different *MAT*B genes) and a *MAT*A x *mat*B^{con} homokaryon (with constitutive *MAT*B activity) the *SC3* gene is repressed (see above), it was hypothesized that interaction between different *MAT*B genes resulting in repression of *SC3* also occurred in dikaryotic cells with closely juxtaposed nuclei. This explains the absence of *SC3* expression in the dikaryotic plectenchyma of the fruit bodies. Possibly the disruption of the binucleate state occurring in incipient aerial hypae interrupts the interaction between different *MAT*B genes. This could cause a switch from the production of SC1, SC4 and SC6 hydrophobins (and other dikaryon-specific proteins) to the production of the SC3 hydrophobin (Ásgeirsdóttir *et al.*, 1995; Wessels *et al.*, 1995).

The hydrophobin genes of *S. commune* may also be subject to environmental regulation but this issue is not yet clear. Light, which is necessary for fruit-body formation in most strains, increased the abundance of the dikaryon-specific hydrophobin mRNAs but decreased the SC3 mRNA level (Wessels *et al.*, 1987; Yli-Mattila *et al.*, 1989b). A similar effect was noted for a low carbon dioxide concentration (Wessels *et al.*, 1987). As explained by Wessels (1992), these effects may be (partly) indirect because light and low carbon dioxide are conducive to the formation of fruit bodies in which the dikaryon-specific mRNAs do not decrease as in the vegetative mycelium. It would seem to be essential to evaluate these effects of the environment in the absence of aerial differentiation, i.e. in shaken cultures.

What are the functions of the hydrophobins SC1, SC4 and SC6? The fruit body begins its development with aggregating hyphae that grow upward from the substratum, followed by inward growth of peripheral hypha forming the pit in which the hymenium develops (Raudaskoski and Viitanen, 1982; van der Valk and Marchant, 1978). Since the genes for these hydrophobins are active from the very beginning of fruit-body initiation, the corresponding products were suggested to play a role in the aggregation process, which plausibly involves some surface component of these hyphae (Wessels *et al.*, 1991a). Whether or not this is true can hopefully be answered by targeted mutations in these hydrophobin genes and by localization of their products. Using cryo-scanning of fully hydrated frozen fruit-body fragments, hyphae making up the plectenchyma could be seen to be embedded in an extracellular matrix traversed by air channels probably serving gas exchange (Ásgeirsdóttir *et al.*, 1995). An antiserum against the dikaryon-specific hydrophilic SC7 localized this protein within the mucilaginous matrix, which binds hyphae together (Schuren *et al.*, 1993a; Ásgeirsdóttir *et al.*, 1995). Experiments with an antiserum directed against SC4 localized this hydrophobin at the interface of the extracellular matrix and the air spaces within the fruit bodies (Wessels *et al.*, 1995), whereas freeze–fracture images showed the presence of rodlets at this interface. Given the property of the SC4 hydrophobin to assemble into an SDS-insoluble film at a water–air interface (this laboratory, unpublished data), this would be an appropriate place for SC4 to assemble into a hydrophobic rodlet layer. One function of the SC4 hydrophobin would thus be to provide the air channels in the xerotolerant fruit bodies with a hydrophobic lining, preventing them from becoming water-soaked during recurrent cycles of drying and wetting.

Hydrophobins have also been found in fruit bodies of *Agaricus bisporus*, the edible white button mushroom (Lugones *et al.*, 1996; Fig. 1). The ABH1 hydrophobin protein is particularly abundant in the closely interwoven hyphae that make up the skin and the veil of the mushroom. As for SC3, ABH1 was found to assemble *in vitro* at hydrophobic–hydrophilic interfaces into a hydrophobic rodlet layer. Since a rodlet layer of ABH1 covers the whole mushroom, ABH1 probably has a function similar to that of the SC3 hydrophobin of *S. commune*, i.e. conferring hydrophobicity to the surface of the fruit body. In addition, rodlets were found

lining air spaces within the fruit bodies, suggesting that ABH1 also provides these air spaces with a hydrophobic lining.

5.3. Formation of Conidia

With a few exceptions, the ascomycetes do not produce large sexual fruit bodies but they do form conspicuous masses of vegetative spores, often conidiospores (conidia) that appear on aerial hyphae (conidiophores), and often at the tips of branches (metulae and phialides). These conidia are generally very hydrophobic, probably an adaptation for aerial dispersal. The molecular genetics of conidiation has been intensively studied in *Aspergillus nidulans* by W.E. Timberlake and co-workers (for reviews, see Timberlake, 1990, 1993). By complementation of developmental mutants (Clutterbuck, 1969), these workers isolated the genes *brlA*, *abaA* and *wetA*. These turned out to be regulatory genes that work in sequence. An unknown signal, generated when the mycelium has attained a certain developmental stage, turns on *brlA*. The *brlA* mutant forms aerial conidiophore stalks that do not differentiate any further structures and continue to grow. *brlA* activates *abaA*, and *abaA* also activates *brlA* in a positive feedback. The *abaA* mutant forms vesicles, metulae and phialides but the latter continue growth without forming conidia. The *abaA* gen activates *wetA*, a gene required for maturation of the conidia. The second approach taken was differential screening of a cDNA library. In this way two hydrophobin genes were found, which, after targeted inactivation, both resulted in a wettable phenotype of the conidia. The first gene, *rodA*, encodes a hydrophobin (Figs 1 and 2) involved in the formation of the rodlet layer on conidia, phialides and metulae (Stringer *et al.*, 1991). The *rodA* mRNA was not found in the developing conidia but accumulates in the phialides. The *rodA* gene is activated by *brlA* but does not need the activities of *abaA* and *wetA*. When the *brlA* gene was placed under control of a promoter that could be induced experimentally (Adams *et al.*, 1988), conidiophores and conidia were produced in submerged culture accompanied by expression of *rodA*, although rodlets were not formed and the hydrophobin probably diffused into the medium (Stringer *et al.*, 1991). Since *rodA* appears under the control of *brlA*, the possibility of *brlA* producing an activator directly acting on *rodA* was investigated by performing an elegant experiment in *Saccharomyces cerevisiae* (Chang and Timberlake, 1993). In this yeast, *brlA*, under control of the *Gal1* promoter, activated *LacZ*, under control of the *rodA* promoter. In this *rodA* promoter, as well as in other genes regulated by *brlA*, multiple copies of the sequence 5'C/A G/A A G G G G/A were found, apparently mediating binding of the *brlA* transcription factor.

The second hydrophobin gene found in *A. nidulans* was *dewA* (Stringer and Timberlake, 1995). DewA was found only on conidia as judged by immunolocalization of an epitope-tagged hydrophobin. However, no rodlets were seen on *rodA*$^-$ *dewA*$^+$ conidia, although DewA contributed to hydrophobicity of the spores.

These conidia were more hydrophobic than *rodA*$^-$ *dewA*$^-$ conidia and *rodA*$^+$ *dewA*$^-$ conidia could be wetted with a solution containing both 0.2% SDS and 50 mM EDTA (but not with solutions containing either of these substances alone). Unlike *rodA*, expression of *dewA* requires activity of *wetA*; activity is only evident after the first spores are produced. This pattern of expression is similar to that of *wA*, encoding a polyketide synthase, and *yA*, encoding a laccase, genes required for pigment production in the spores, but likewise not transcribed in the spores. These proteins are apparently all transported into the developing conidia at a late stage during their maturation, while the product of the *rodA* gene is incorporated earlier (Stringer and Timberlake, 1995). There were also rodlets found on stalks and vesicles of conidiophores (Stringer *et al.*, 1991), which apparently were not the product of *rodA* or *dewA*. So, other hydrophobin genes probably exist in *A. nidulans*. Since *rodA* and *dewA* are only locally expressed in the conidiophores, the hydrophobin-like proteins found in the culture medium of wild-type *A. nidulans* (de Vries *et al.*, 1993) might be encoded by other hydrophobin genes. However, it is also possible that, in submerged cultures, *rodA* and *wetA* are induced under conditions of C or N starvation as are the rodlet genes of *Neurospora crassa* (Sokolovsky *et al.*, 1992) and *Magnaporthe grisea* (Talbot *et al.*, 1993).

Another conidiating system in which a hydrophobin has been implicated is *N. crassa*. The gene for this hydrophobin was independently identified by Bell-Pedersen *et al.* (1992) as a gene (*ccg2*) controlled by the circadian rhythm in this fungus (mRNAs reaching high values in the subjective morning) and by Lauter *et al.* (1992) as a gene (*bli-7*) induced in dark-grown cultures by blue light. Both groups showed that the gene complemented the previously isolated *eas* mutant, which has easily wettable conidia (Selitrennikoff, 1976) and lacks rodlets (Beever and Dempsey, 1978). Because *ccg-2* and *bli-7* are allelic to *eas*, the gene is referred to as *eas* (Figs 1 and 2).

It appears that the original *eas* mutant is leaky; expression of mRNA could be detected and some rodlets were found on the spores of the *eas* mutant (Bell-Pedersen *et al.*, 1992). There is nothing wrong with the coding sequence, but an insertion was found between –1000 and –1500 base pairs upstream of the transcription start point (Lauter *et al.*, 1992). It is somewhat surprising to find a *cis*-regulatory element so far upstream. However, in a promoter analysis of *eas*, Kaldenhoff and Russo (1993) indeed found that a region located between –1498 bp and –1079 bp acted as a positive regulatory element necessary for light activation, and for activation by carbon and nitrogen starvation. Deletion of this region led to complete inactivation of the gene. In addition, they found a positive element for light induction between –429 bp and –380 bp, and a negative regulatory element in the region –595 bp and –429 bp that appeared to inhibit the adjacent light-sensitive element. In surface cultures, *eas* seems to be precisely regulated (Lauter *et al.*, 1992). Its mRNA becomes abundant at a precise point of conidiospore production but is not found in the conidia; as in *A. nidulans* conidiogenesis, the rodlet protein is apparently transported into the spore. However, the *eas* gene is

also expressed in *acon-2* and *acon-3* mutants that cannot conidiate. A mutation in *fl*, however, blocks expression of *eas*. Since this mutation also produces aerial hyphae that are easily wettable, the *fl* gene may regulate unknown hydrophobin genes in this organism, in addition to *eas*.

The mRNA of *eas* is also produced when *N. crassa* is grown in submerged culture under conditions of nitrogen starvation that induce conidiation, even to estimated levels of 10–25% of polyA$^+$mRNA, 50–100 times higher than in surface cultures (Sokolovsky *et al.*, 1992). Also, the *MPG1* gene, responsible for hydrophobicity of conidia of *Magnaporthe grisea*, is transcribed in submerged growing mycelium during starvation for carbon or nitrogen (Talbot *et al.*, 1993). Although it has not yet been shown that any of these hydrophobins produce rodlets by interfacial self-assembly, presumably the hydrophobins were secreted into the medium and not assembled on any conidia that formed submerged.

5.4. Pathogenesis

The surfaces of plants and animals can be extremely hydrophobic. Most plant and arthropod surfaces show water contact angles above 100° up to 170°, while the human skin was also found to be rather hydrophobic with water contact angles of 100° (Wösten *et al.*, 1995). However, it should be realized that the wettability of these surfaces is caused not only by their chemical composition but is strongly influenced by topographical structures, for instance, owing to wax morphology and epidermal ridges (Troughton and Hall, 1967; Holloway, 1970; Netting and von Wettstein-Knowles, 1973). These studies have indicated that water contact angles of smooth surfaces could be raised from 90° to 165° solely by the presence of surface roughness. Since water contact angles measured on smooth surfaces of Teflon and Parafilm do not exceed 110° (Wösten *et al.*, 1994a), it is likely that all high-contact angles measured on natural surfaces are partly due to structural features of these surfaces.

The extensive literature on adhesion of fungi to the hydrophobic surfaces of plants and arthropod cuticles has been reviewed (Boucias and Pendland, 1991; Nicholson and Epstein, 1991; Mendgen and Deising, 1993; Clement *et al.*, 1994; Jones, 1994). It is generally thought that hydrophobic interactions occur between the hydrophobic surfaces of airborne spores and the host surface. At least in cases where rodlets are observed on the spores, hydrophobins are probably involved. Also, active adhesion of spores was observed. For instance, upon hydration, spores of the rice blast fungus, *Magnaporthe grisea*, expel a preformed material from the site of future germ-tube formation by which they tightly adhere to hydrophobic surfaces (Hamer *et al.*, 1988). In addition, the germ tube and the appressorium must adhere tightly to the hydrophobic surface because the infection peg, which penetrates the epidermis, arises from the latter structure. Some fungi can do this by

sheer mechanical force, as demonstrated a century ago by Miyoshi (1895) and more recently for *M. grisea* by Howard *et al.* (1991).

During nutrient deprivation, the insect pathogen *Metarhizium anisopliae* produced haustoria and cuticle-degrading enzymes *in vitro*, and at the same time abundantly transcribed the hydrophobin gene *ssg A* (St Leger *et al.*, 1992). These authors suggested that the SSGA hydrophobin (Figs 1 and 2) is involved in building the wall of the haustorium and could assist in hydrophobic attachment to the cuticular surface. Talbot *et al.* (1993) detected abundant transcription of the hydrophobin gene *MPG1* (Figs 1 and 2) during infection of rice plants with *Magnaporthe grisea*. *MPG1* mRNA is highly abundant very early in infection, concomitant with appressorium formation, while a second peak of *MPG1* mRNA occurs during symptom development. They also performed a gene disruption and observed that the *Mpg1* mutants had a reduced ability to cause disease symptoms, which appears to result from an impaired ability to undergo appressoria formation. Since appressorium formation is triggered in this case by a hydrophobic surface (Hamer *et al.*, 1988), it was assumed that in the *Mpg1* mutant, in the absence of a hydrophobin-mediated contact between the fungal wall and the inducing surface, a morphogenetic signal for appressorium formation is not generated. However, it was recently shown (Talbot *et al.*, 1996) that wild-type and mutant germlings adhere equally well to Teflon, but that the latter grow longer hyphae and make fewer appressoria. It would thus seem that the effect of the MPG1 hydrophobin is very specific for generating the signal for appressorium formation and that another hydrophobin may be responsible for attachment of the germlings to the hydrophobic surface. Remarkably, the MPG1 hydrophobin was shown to be responsible also for generating the hydrophobic rodlet layer on the conidia of this fungus.

Most germlings of plant pathogenic fungi adhere best to hydrophobic surfaces, some clearly exhibit greatest adhesion to hydrophilic surfaces, and a few adhere to both types of surfaces (Nicholson and Epstein, 1991; Nicholson and Kunoh, 1995). Terhune and Hoch (1993) demonstrated that *Uromyces appendiculatus* urediospore germlings exhibit best adhesion to hydrophobic substrata. A range of surface hydrophobicities was prepared by treating glass with a variety of silanes. The most hydrophobic surface, determined by measuring surface wettability, allowed the greatest adhesion of germlings to the substrate. Also, small topographic features of the surface were most inducive to appressoria formation when these were hydrophobic. These observations closely parallel the assembly and adhesion of the SC3 hydrophobin of *Schizophyllum commune* to a continuous hydrophobicity gradient obtained by coating glass with dichlorodimethylsilane (Wösten *et al.*, 1995).

How could secretion of a hydrophobin attach a hypha to a hydrophobic surface? Wösten *et al.* (1994a) found that the SC3 hydrophobin assembles into an SDS-insoluble monolayer on hydrophobic surfaces, making these surfaces wettable. Assuming that the hydrophilic side of the assembled hydrophobin strongly binds to hydrophilic polysaccharide components of the wall, secretion of the SC3

hydrophobin by a hypha of *Schizophyllum commune*, forced to grow over Teflon, would thus be expected to glue the hypha to the Teflon. This was shown to be the case and SC3 could be immunolocalized between the hypha and the Teflon. Moreover, attachment of hyphae to the Teflon was reduced in a strain with a targeted disruption of the *SC3* gene. However, the wild-type strain did not adhere to the Teflon when immersed in water or liquid medium (this laboratory, unpublished data), although, owing to secretion of SC3 in the surrounding liquid, the Teflon became hydrophilic. It is, therefore, conceivable that a pathogenic fungus first wets a surface with a hydrophobin and then tightly adheres to the hydrophilic side of the amphipathic hydrophobin film on a leaf or insect cuticle surface by means of a hydrophilic mucilage. Mucilages have often been seen as apparently attaching the hypha or appressorium to the host surface (Nicholson and Epstein, 1991).

Figure 5 shows a diagram of the two possible ways for a hypha to adhere to a hydrophobic surface by means of a hydrophobin. Figure 5a depicts a situation as observed for hydrophobin-mediated attachment of a *S. commune* hypha to Teflon under dry conditions. Figure 5b depicts a possible means of attachment for a pathogenic fungus under humid conditions, implicating both a hydrophobin and a hydrophilic mucilage. It should be emphasized that the mucilage and the hydrophobin could be simultaneously secreted and that there might be specific lectin-like interactions between the hydrophilic side of the hydrophobin film and the mucilage. The participation of lectins has been suggested by studies on adhesion of *Magnaporthe grisea* to artificial surfaces (Xiao *et al.*, 1994). If the involved hydrophobins have lectin-like activity, this would explain why the mucilage cannot provide for adherence to any hydrophilic surface but that a hydrophobic surface is required for attachment of the fungus. The beauty of this system is its simplicity. The hydrophobicity of the surface would be sensed by a secreted protein, which by self-assembly serves as an adhesive with an amphipathic character gluing two incompatible surfaces together.

As mentioned earlier (Section 4.2), cerato-ulmin produced by *Ophiostoma ulmi* has been implicated in causing Dutch elm disease. The reason for its assumed role as a phytotoxin was mainly based on correlative evidence and on the ability of isolated cerato-ulmin to cause the wilting syndrome in plant cuttings (Richards, 1993). A compelling reason for assuming a role in wilting was also the occurrence of similar plugging phenomena in the xylem of elm after infection with *O. ulmi* and application of cerato-ulmin to elm cuttings (Takai and Hiratsuka, 1984). It was plausible to assume that wilting was initiated by assembly of the surface-active cerato-ulmin around air bubbles arising in the xylem fluid under negative pressure (Russo *et al.*, 1982). However, strains of *O. nova-ulmi* carrying the pleiotropic mutation cu^- did not produce cerato-ulmin, had no aerial hyphae but were nevertheless virulent (Brasier *et al.*, 1995). Disruption of the gene for cerato-ulmin recently showed that indeed cerato-ulmin is not involved in virulence of the fungus (Bowden *et al.*, 1996). Vascular discoloration and foliar wilting in elm seedlings

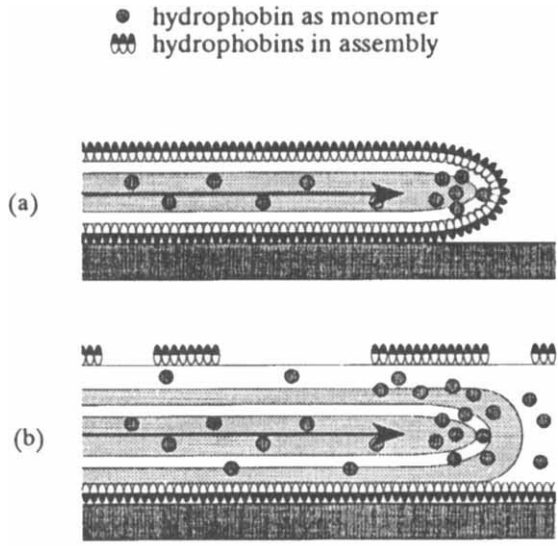

Figure 5 Proposed models for the attachment of hyphae to hydrophobic surfaces. Symbols are as in Fig. 4. In (a) the hypha is growing in air over the hydrophobic substrate and secreted hydrophobin monomers assemble directly at the hyphal surface exposed to air, and between the wall and the hydrophobic substratum, firmly attaching the hypha. This was observed for hyphae of *Schizophyllum commune* forced to grow over dry Teflon (Wösten *et al.*, 1994a). In (b) it is assumed that free hydrophobin monomers are secreted into the liquid or mucilage surrounding the hyphae, and that the hydrophobin, by assembling on the hydrophobic surface, creates a hydrophilic surface to which the hypha can attach by means of the mucilage, which serves as a hydrophilic adhesive, possibly binding specifically to the primed surface. (From Wessels, 1996, with permission from the publisher.)

are the same for plants inoculated with an aggressive strain of *O. ulmi* producing abundant cerato-ulmin and the same strain with the targeted mutation that does not produce cerato-ulmin. Since the mutant produces few aerial hyphae and is easily wettable, cerato-ulmin is probably involved in aerial growth of this fungus. However, it remains possible that *O. ulmi* produces another hydrophobin under conditions prevailing in the tree that is responsible for disease symptoms and that cerato-ulmin is just mimicking the effects of this hydrophobin. It should also be noted that a similar Class II hydrophobin produced by *Cryphonectria parasitica*, cryparin (CRYP; Figs 1 and 2), has not been considered to be a phytotoxin (Carpenter *et al.*, 1992) and that non-pathogenic fungi produce similar hydrophobins (see Fig. 1).

Hydrophobicity of the fungal surface has been implicated in fungal infections of humans (Hazen, 1990). This is a vast field that becomes increasingly important because the incidence of immunocompromised patients infected by pathogenic and

opportunistic fungi is rapidly increasing. The involvement of hydrophobins in the infection process has not yet been reported. The availability of the *rodA* gene of *Aspergillus nidulans* has enabled cloning of the corresponding gene from the pathogenic *A. fumigatus* (Parta *et al.*, 1994; Thau *et al.*, 1994). However, disruption of the gene, leading to formation of wettable conidia without rodlets, did not lead to a decrease in pathogenicity in mice (Thau *et al.*, 1994). The importance of the hydrophobic rodlet layer probably lies in aerial dispersal of the fungus but other members of the hydrophobin family may still prove to be of importance in pathogenesis. Hydrophobins may play a role in attachment of invading hyphae to host cells or hydrophobic implants. In addition, because invading fungi may release large quantities of specific circulating hydrophobins, these might be used for diagnosis of the mycosis with specific antibodies.

Since hydrophobins are small, abundantly secreted proteins that fulfil important roles in fungal development, it is conceivable that plants sense the presence of fungi by having receptors for these proteins. Hydrophobins or derived proteins could thus act as elicitors of the defence response in plants after infection with pathogenic fungi (Wessels, 1994). Known peptide elicitors have some resemblance to hydrophobins, particularly in being rich in cysteine residues, and it has been suggested that these elicitors are structurally related to hydrophobins (Sticklen and Bolyard, 1994; Templeton *et al.*, 1994).

5.5. Symbiosis

Symbiosis of fungi with other organisms always involves intimate contact of the fungal surface with host tissue. In two cases hydrophobins have now been implicated. F. Martin's group has studied molecular events occurring during formation of ectomycorrhiza (Martin *et al.*, 1995). During formation of the ectomycorrhizal mantle in the association between *Pisolithus tinctorus* and the roots of *Eucalyptus globulus*, they found high expression of two genes encoding hydrophobins (*HydPt-1* and *HydPt-2*) (Tagu *et al.*, 1996, and Fig. 1). They speculate that these hydrophobins might be involved in aggregation of the hyphae, forming the hyphal tissue around the root, or aid in attachment of hyphae to the root during initial colonization (Martin *et al.*, 1995). Alternatively, they may create the hydrophobic surface of the fungal mantle.

Another case of symbiosis in which hydrophobins have been implicated, but not yet isolated, concerns lichens (Honneger, 1993). This is particular interesting because lichens are fungi that have reached the ultimate stage in emergent growth. They always live above the substrate, retrieving organic material from intimately associated algae and cyanobacteria that assimilate carbon dioxide and dinitrogen from the air. Some 20% of the fungi are lichenized and these lichens are the only "vegetation" in about 8% of the land area, particularly tundra and high mountainous areas (Honneger, 1991). Because of their habitat, they are exposed to extreme

cycles of wetting and drying, and temperature changes. The fungal hyphae were seen to make simple contacts with algae, the whole being ensheathed by a matrix decorated on the outside with a hydrophobic rodlet layer, where these associations contacted the air spaces within the thallus (Honneger, 1991). Although a hydrophobin composition of this rodlet was not established, spontaneous self-assembly of secreted hydrophobins could explain how this rodlet layer could extend from the fungus over the algal surface. The possible function of the rodlet layer (and other hydrophobic substances) lining the air spaces could be all-important for the existence of this symbiosis (Honneger, 1993; Honneger and Peter, 1994). First, the layer would collectively shield the apoplast of fungus and photosymbiont from the air spaces, permitting apoplastic transport of water and solutes to and from the symbiont. Second, the lining of the air spaces with a hydrophobic rodlet layer would permit optimal gas exchange and prevent the air channels from becoming soaked with water during wetting after a dry period.

6. TECHNOLOGY

Although this review deals principally with roles of hydrophobins in biological systems, the novel properties of hydrophobins as discussed in Section 4 immediately raise the possibilities of application of hydrophobins in technology. Materials science has a considerable interest in self-assembling molecules from nature (Service, 1994). The abilities of hydrophobins to self-assemble at interfaces into insoluble films are not parallelled by any other known protein and therefore it is likely that applications will be found. In fact, the roles of hydrophobins in fungal growth and development, as discussed in Section 5, give direction to the kind of applications that can be thought of: *"natura artis magistra"*. At the moment only the imagination limits the potential application of hydrophobins.

One condition for the successful application of hydrophobins is that they can be cheaply produced in quantity. As mentioned before, hydrophobins are among the most abundant proteins secreted by fungi. Class I hydrophobins appear to be the most promising for application because of the stability of the assembled films. These hydrophobins appear to be particularly abundant in the culture medium of members of the basidiomycetes. For instance, it has been calculated that, in 4-day-old cultures of *Schizophyllum commune*, about 15% of the ^{35}S incorporated into protein goes into synthesis of the SC3 hydrophobin (de Vries *et al.*, 1993), while up to 20 mg of SC3 can be easily purified from one litre of culture medium by a simple procedure based on the extraordinary properties of the protein (4.1). Strain selection and optimizing culture conditions could probably enhance the yield as could molecular genetic methods, such as increasing gene dose and heterologous production in fungi in common use in the fermentation industry.

On the other hand, it should be realized that quantities needed for certain applications may be small. This is expected from the use that nature makes of an "expensive" product as a protein for changing the wettability of surfaces. Indeed, the very nature of the assembled amphipathic film requires that it is present as a monolayer. The thickness of this monolayer is only about 10 nm and thus very little hydrophobin is required to achieve a drastic change in wettability. From the number of molecules of SC3 adsorbed to Teflon (Wösten *et al.*, 1994a), it can be calculated that about 1.5 mg SC3 hydrophobin suffices to coat 1 m^2 of Teflon surface with the effect of decreasing the hydrophobicity of this surface from 110° to 48° water contact angles.

Another important lesson from nature is that a fungal species makes different hydrophobins for different purposes. For instance, in *S. commune* an SC3 film appears to coat aerial hyphae and to confer water-repellent properties to these structures, whereas air channels in fruit bodies are lined with an assemblage of SC4 hydrophobin. One wonders whether possible biophysical differences in the properties of these films are tuned to different functions, especially because hydrophobins from widely different species may be more closely related than different hydrophobins within one species (Table 1). This is certainly a point to be considered in any biomimetics before resorting to genetic engineering to tailor a hydrophobin for a specific purpose.

Of the many possible applications of hydrophobins, a few of the more obvious ones are listed below.

1. Hydrophobins may be used in tissue engineering (Hubbell, 1995), particularly for coating hydrophobic surfaces to increase their biocompatibility. As already noted, the attachment of the hydrophobin film to hydrophobic surfaces is very strong and the change in surface wettability significant. For instance, the hydrophobins may be used to enhance the biocompatibility of medical implants, including artificial blood vessels and surgical instruments.
2. Hydrophobins may be used as an intermediate to attach cells, proteins, such as antibodies, and small ligands to hydrophobic surfaces, as in biosensors. For instance, researchers at the Department of Bioprocessing and Biomonitoring of TNO, Zeist, The Netherlands, in collaboration with our group, have shown that the SC3 hydrophobin readily coats a hydrophobic gold surface. At the exposed hydrophilic side of the SC3 film, mannose residues can be oxidized with periodic acid without disturbing the binding to the gold, while the generated aldehyde groups can be easily coupled to amino groups of a protein by a Schiff-base reaction.
3. Hydrophobic solids or liquids (oils) can be dispersed in water by coating with a hydrophobin (Wösten *et al.*, 1994a). Oil vesicles coated with a hydrophobin film may be useful for delivery of lipophilic drugs. A glycosylated hydrophobin, such as SC3, would permit easy attachment of targeting antibodies to the outside of such vesicles.

4. Hydrophobins have unknowingly been ingested by humans for millennia when eating mushrooms and fungus-fermented foods. At least when derived from GRAS fungi (Generally Regarded As Safe), hydrophobins can be considered safe for consumption and can be used in foods and drinks.
5. The property of hydrophobins to coat a surface with a very thin layer (about 10 nm) that nevertheless dramatically changes the nature of this surface promises the use of these proteins in nanotechnology as defined by Thomas (1995).

7. CONCLUSIONS

Hydrophobins are a novel class of small secreted cysteine-rich proteins of fungi that assemble into amphipathic films when confronted with hydrophilic–hydrophobic interfaces. Some hydrophobins form unstable, others extremely stable, amphipathic films. By assembling at a wall–air interface some have been shown to provide for a hydrophobic surface, which has the ultrastructural appearance of rodlets as on aerial hyphae and spores. Some hydrophobins have been shown to assemble into amphipathic films at interfaces between water and oils, or hydrophobic solids, and may be involved in adherence phenomena. It appears that hydrophobins are among the most abundantly produced proteins of fungi, and individual species may contain several genes producing divergent hydrophobins, possibly tailored for specific purposes. Hydrophobins have now been implicated in various developmental processes, such as formation of aerial hyphae, fruit bodies and conidia, and may play essential roles in fungal ecology, including spore dissemination, pathogenesis and symbiosis. The surfactive properties of hydrophobins and the ability of some of them to form very stable insoluble amphipathic films, which change the wettability of surfaces, also makes them good candidates for technical applications.

ACKNOWLEDGEMENTS

The author is grateful to all members of his laboratory for stimulating discussions and for permission to quote unpublished results. He is particularly indebted to Dr Han A.B. Wösten for carefully reading the manuscript and to colleagues in other laboratories who were willing to communicate unpublished results.

REFERENCES

Adams, T.H., Boylan, M.T. and Timberlake, W.E. (1988) *brlA* is necessary and sufficient to direct conidiophore development in *Aspergillus nidulans*. *Cell* **54**, 353–362.

Andersen, N.H., Cao, B., Rodrígez-Romero, A. and Arreguin, B. (1993) Hevein: NMR assignment of solution-state folding for the agglutinin-toxin motif. *Biochemistry* **32**, 1407–1422.

Aronson, A.I. and Fitz-James, P. (1976) Structure and morphogenesis of the bacterial spore coat. *Bacteriol. Rev.* **40**, 360–402.

Ásgeirsdóttir, S.A., van Wetter, M.A. and Wessels, J.G.H. (1995) Differential expression of genes under control of the mating-type genes in the secondary mycelium of *Schizophyllum commune*. *Microbiology* **141**, 1281–1288.

Banham, A.H., Asante-Owusu, R.N., Göttgens, B., Thompson, S.A.J., Kingsnorth, C.S., Mellor, E.J.C. and Casselton, L.A. (1995) An N-terminal dimerization domain permits homeodomain proteins to choose compatible partners and initiate sexual development in the mushroom *Coprinus cinereus*. *Plant Cell* **7**, 773–783.

Beever, R.E. and Dempsey, G. (1978) Function of rodlets on the hyphae of fungal spores. *Nature* **272**, 608–610.

Beever, R.E., Redgewell, R.J. and Dempsey, G. (1979) Purification and chemical characterization of the rodlet layer of *Neurospora crassa* conidia. *J. Bacteriol.* **140**, 1063–1070.

Bell-Pedersen, D., Dunlap, J.C. and Loros, J.J. (1992) The *Neurospora* circadian clock-controlled gene, *ccg*-2, is allelic to *eas* and encodes a fungal hydrophobin required for formation of the conidial rodlet layer. *Genes Dev.* **6**, 2382–2394.

Bidochka, M.J., St Leger, R.J., Joshi, L. and Roberts, D.W. (1995a) The rodlet layer from aerial and submerged conidia of the entomopathogenic fungus *Beauveria bassiana* contains hydrophobin. *Mycol. Res.* **99**, 403–406.

Bidochka, M.J., St Leger, R.J., Joshi, L. and Roberts, D.W. (1995b) An inner cell wall protein (cwp1) from conidia of the entomopathogenic fungus *Beauveria bassiana*. *Microbiology* **141**, 1075–1080.

Bohlmann, H. and Apel, K. (1991) Thionins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 227–240.

Bolyard, M.G. and Sticklen, M.B. (1992) Expression of a modified Dutch elm disease toxin in *Escherichia coli*. *Mol. Plant–Microbe Int.* **5**, 520–524.

Boucias, D.G. and Pendland, J.C. (1991) Attachment of mycopathogens to cuticle. The initial events of mycoses in arthropode hosts. In: *The Fungal Spore and Disease Initiation in Plants and Animals* (G.T. Cole and H.C. Hoch, eds), pp. 101–127. Plenum Press, New York, London.

Bowden, C.G., Hintz, W.E., Jeng, R., Hubbes, M. and Horgen, P.A. (1993) Isolation and characterization of the cerato-ulmin toxin gene of the Dutch elm disease pathogen, *Ophiostoma ulmi*. *Curr. Genet.* **25**, 323–329.

Bowden, C.G., Smalley, E., Guries, R.P., Temple, B., Hubbes, M. and Horgen, P.A. (1996) Lack of association between cerato-ulmin production and virulence in *Ophiostoma nova-ulmi*. *Mol. Plant–Microbe Int.* (in press).

Brasier, C.M., Takai, S., Nordin, J.H. and Richards, W.C. (1990) Differences in cerato-ulmin production between the EAN, NAN and non-aggressive subgroups of *Ophiostoma ulmi*. *Plant Pathol.* **39**, 231–236.

Brasier, C.M., Kirk, S.A. and Tegli, S. (1995) Naturally occurring non-cerato-ulmin producing mutants of *Ophiostoma novo-ulmi* are pathogenic but lack aerial mycelium. *Mycol. Res.* **99**, 436–440.

Bronchart, R. and Demoulin, V. (1971) Ultrastructure de la paroi des basidiospores de *Lycoperdon* et de *Scleroderma* (Gastéromycètes) comparée à celle de quelques autres spores de champignons. *Protoplasma* **72**, 179–189.

Busscher, H.J., van Pelt, A.W.J., de Boer, P., de Jong, H.P. and Arends, J. (1984) The effect of surface roughening of polymers on measured contact angles of liquids. *Colloids Surf.* **9**, 319–331.

Carpenter, C.E., Mueller, R.J., Kazmierczak, P., Zhang, L., Villalon, D.K. and Van Alfen, N.K. (1992) Effect of a virus on accumulation of a tissue-specific cell-surface protein of the fungus *Cryphonectria (Endothia) parasitica*. *Mol. Plant–Microbe Int.* **4**, 55–61.

Casselton, L.A. and Kües, U. (1994) Mating-type genes in homobasidiomycetes. In: *The Mycota*, Vol. I, *Growth, Differentiation and Development* (J.G.H. Wessels and F. Meinhardt, eds), pp. 307–321. Springer Verlag, Berlin.

Castonguay, Y., Laberge, S., Nadeau, P and Vézina, L.-P. (1994) A cold-induced gene from *Medicago sativa* encodes a bimodular protein similar to developmentally regulated proteins. *Plant Mol. Biol.* **24**, 799–804.

Cavalier-Smith, T. (1993) Kingdom protozoa and its 18 phyla. *Microbiol. Rev.* **57**, 953–994.

Chang, Y.C. and Timberlake, W.E. (1993) Identification of *Aspergillus brlA* response elements (BREs) by genetic selection in yeast. *Genetics* **133**, 29–38.

Claverie-Martin, F., Diaz-Torres, M.R. and Geoghegan, M.G. (1986) Chemical composition and electron microscopy of the rodlet layer of *Aspergillus nidulans* conidia. *Curr. Microbiol.* **14**, 221–225.

Clement, J.A., Porter, R., Butt, T.M. and Beckett, A. (1994) The role of hydrophobicity in attachment of urediniospores and sporelings of *Uromyces viciae-fabae*. *Mycol. Res.* **98**, 1217–1228.

Clutterbuck, A.J. (1969) A mutational analysis of conidial development in *Aspergillus nidulans*. *Genetics* **63**, 317–327.

Cole, G.T. and Hoch, H.C., eds (1991) *The Fungal Spore and Disease Initiation in Plants and Animals*. Plenum Press, New York.

Cole, G.T., Sekiya, M., Kasai, R.L. Yokoyama, T. and Nozawa, Y. (1979) Surface ultrastructure and chemical composition of the cell walls of conidial fungi. *Exp. Mycol.* **3**, 132–156.

de Groot, P.W.J., Schaap, P.J., Sonnenberg, A.S.M., Visser, J. and van Griensven, L.J.L.D. (1996) The *Agaricus bisporus hypA* gene encodes a hydrophobin and specifically accumulates in peel tissue of mushroom caps during fruit body development. *J. Mol. Biol.* **257**, 1008–1018.

de Vries, O.M.H., Fekkes, M.P., Wösten, H.A.B. and Wessels, J.G.H. (1993) Insoluble hydrophobin complexes in the walls of *Schizophyllum commune* and other filamentous fungi. *Arch. Microbiol.* **159**, 330–335.

Dons, J.J.M., Springer J., de Vries S.C. and Wessels, J.G.H. (1984) Molecular cloning of a gene abundantly expressed during fruit-body initiation in *Schizophyllum commune*. *J. Bacteriol.* **157**, 802–805.

Drenth, J., Low, B.J., Richardson, J.S. and Wright, C.S. (1980) The toxin-agglutinin fold: a new group of small protein structures organized around a four-disulphide core. *J. Biol. Chem.* **255**, 2652–2655.

Elwing, H., Welin, S., Askendal, A., Nilssom, U. and Lundström, L. (1987) A wettability gradient method for studies of macromolecular interactions at the liquid–solid interface. *J. Colloid Interface Sci.* **119**, 203–210.

Fisher, D.J, Brown, G.A. and Holloway, P.J. (1978) Influence of growth medium on surface and wall lipid of fungal spores. *Phytochemistry* **17**, 85–89.

Gerin, P.A., Asther, M., Sleytr, U.B. and Rouxhet, P.G. (1994) Detection of rodlets in the outer wall region of conidiospores of *Phanerochaete chrysosporium*. *Can. J. Microbiol.* **40**, 412–416.

Hamer, J.E., Howard, R.J., Chumleym, F.G. and Valent, B. (1988) A mechanism for surface attachment in spores of a plant pathogenic fungus. *Science* **239**, 288–290.

Harley, J.L. and Smith, S.E. (1983) *Mycorrhizal Symbiosis*. Academic Press, London, New York.

Hashimoto, T., Wu-Yuan, C.D. and Blumenthal, H.J. (1976) Isolation and characterization

of the rodlet layer of *Trichophyton mentagrophytes* microconidial wall. *J. Bacteriol.* **127**, 1543–1549.

Hazen, K.C. (1990) Cell surface hydrophobicity of medically important fungi, especially Candida species. In: *Microbial Cell Surface Hydrophobicity* (R.J. Doyle and M. Rosenberg, eds), pp. 249–95. American Society of Microbiology, Washington, DC.

Hazlett, R.D. (1992) On surface roughness effects in wetting phenomena. *J. Adhesion Sci. Technol.* **6**, 625–633.

Hess, W.M., Sassen, M.M.A. and Remsen, C.C. (1968) Surface characteristics of Penicillium conidia. *Mycologia* **60**, 290–303.

Higgins, D.G. and Sharp, P.M. (1988) Clustal: a package for performing multiple sequence alignments on a microcomputer. *Gene* **73**, 237–244.

Hobot, J.A. and Gull, K. (1981) Structure and biochemistry of the spore surface of *Syncephalastrum racemosum*. *Curr. Microbiol.* **5**, 183–185.

Holloway, P.J. (1970) Surface factors affecting the wetting of leaves. *Pestic. Sci.* **1**, 156–163.

Holt, S.C. and Leadbetter, E.R. (1969) Comparative ultrastructure of selected aerobic spore forming bacteria. *Bacteriol. Rev.* **33**, 346–378.

Honneger, R. (1991) Functional aspects of the lichen symbiosis. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **42**, 553–578.

Honneger, R. (1993) Developmental biology of lichens. *New Phytol.* **125**, 659–677.

Honneger, R. and Peter, M. (1994) Routes of solute translocation and location of water in heteromerous lichens visualized with cryotechniques in light and electron microscopy. *Symbiosis* **16**, 167–186.

Howard, R.J., Ferrari, M.A., Roach, D.H. and Money, N.P. (1991) Penetration of hard substrates by a fungus employing enormous turgor pressures. *Proc. Natl Acad. Sci. U.S.A.* **88**, 11281–11284.

Hubbell, J.A. (1995) Biomaterials in tissue engineering. *Biotechnology* **13**, 565–576.

Huh, C. and Mason, S.G. (1977) Effects of surface roughness on wetting (theoretical) *J. Colloid Interf. Sci.* **60**, 11–38.

Jennings, D.H. (1984) Water flow through mycelia. In: *The Ecology and Physiology of Fungal Mycelia* (D.H. Jennings and A.D.M. Rayner, eds), pp. 143–164. Cambridge University Press, Cambridge.

Jennings, D.H. (1994) Translocation in mycelia. In: *The Mycota*, Vol. I, *Growth, Differentiation and Development* (J.G.H. Wessels and F. Meinhardt, eds), pp. 163–173. Springer Verlag, Berlin.

Jones, E.B.G. (1994) Fungal adhesion. *Mycol. Res.* **98**, 961–981.

Kaldenhoff, R. and Russo, V.E.A. (1993) Promoter analysis of the *bli-7/eas* gene. *Curr. Genet.* **24**, 394–399.

Kües, U. and Casselton, L.A. (1992) Fungal mating type genes – regulators of sexual development. *Mycol. Res.* **96**, 993–1006.

Kyte, J. and Doolittle, R.F. (1982) A simple method for displaying the hydropathy character of a protein. *J. Mol. Biol.* **157**, 105–132.

Lauter, F.-R., Russo, V.E.A. and Yanovsky, C. (1992) Developmental and light regulation of *eas*, the structural gene for the rodlet protein of *Neurospora*. *Genes Dev.* **6**, 2373–2381.

Lora, J.M., de la Cruz, J., Benitez, T., Llobell, A. and Pintor-Toro, J.A. (1994) A putative catabolite-repressed cell wall protein from the mycoparasitic fungus *Trichoderma harzianum*. *Mol. Gen. Genet.* **242**, 461–466.

Lugones, L.G., Bosscher, J., Scholtmeyer, K., de Vries, O.M.H. and Wessels, J.G.H. (1996) An abundant hydrophobin (ABH1) forms hydrophobic rodlet layers in *Agaricus bisporus* fruiting bodies. *Microbiology* **142**, 1321–1329.

Martin, F., Laurent, P., de Cavalho, D., Burgess, T., Murphy, P., Nehls, U. and Tagu, D. (1995)

Fungal gene expression during ectomycorrhiza formation. *Can. J. Bot.* **73**, (Suppl. 1), S541–S547.

Mendgen, K. and Deising, H. (1993) Infection structures of fungal plant pathogens – a cytological and physiological evaluation. *New Phytol.* **124**, 193–213.

Miyoshi, M. (1895) Die Durchbohrung von Membranen durch Pilzfäden. *Jahrb. wiss. Bot.* **28**, 269–289.

Moore, R.T. (1985) The challenge of the dolipore/parenthosome septum. In: *Developmental Biology of Higher Fungi* (D. Moore, L.A. Casselton, D.A. Wood and J.C. Frankland, eds), pp. 175–212. Cambridge University Press, Cambridge.

Mulder, G.H. and Wessels, J.G.H. (1986) Molecular cloning of RNAs differentially expressed in monokaryons and dikaryons of *Schizophyllum commune*. *Exp. Mycol.* **10**, 214–227.

Muñoz, G.A., Agosin, E., Cotoras, M., San Martin, R. and Volpe, D. (1995) Comparison of aerial and submerged spore properties for *Trichoderma harzianum*. *FEMS Microbiol. Lett.* **125**, 63–70.

Nakari-Setälä, T., Aro, N., Kalkkinen, N., Alatalo, E. and Penttilä, M. (1996) Genetic and biochemical characterization of the *Trichoderma reesei* hydrophobin HFB1. *Eur. J. Biochem.* **235**, 248–255.

Nespoulous, C., Huet, J.-C. and Pernollet, J.-C. (1992) Structure–function relationships of α and β elicitins, signal proteins involved in the plant-*Phytophthora* interaction. *Planta* **186**, 551–557.

Netting, A.G. and von Wettstein-Knowles, P. (1973) The physico-chemical basis of leaf wettability in wheat. *Planta* **114**, 293–309.

Nicholson, R.L. and Epstein, L. (1991) Adhesion of fungi to the plant surface: prerequisite for pathogenesis. In: *The Fungal Spore and Disease Initiation in Plants and Animals* (G.T. Cole and H.C. Hoch, eds), pp. 3–23. Plenum Press, New York, London.

Nicholson, R. and Kunoh, H. (1995) Early interactions, adhesion, and establishment of the infection count in *Erysiphe graminis*. *Can. J. Bot.* **73** (Suppl.), S609–S615.

Noordmans, J. and Busscher, H.J. (1991) The influence of droplet volume and contact angle on liquid surface tension measurements by axisymmetric drop shape analysis-profile (ADSA-P) *Colloids Surf.* **58**, 239–249.

Parta, M., Chang, Y., Rulong, S., Pinto-DaSilva, P. and Kwon-Chung, K.J. (1994) HYP1, a hydrophobin gene from *Aspergillus fumigatus* complements the *rodletless* phenotype in *Aspergillus nidulans*. *Inf. Immun.* **62**, 4389–4395.

Raikhel, N.V., Lee, H.-I. and Broekaert, W.F. (1993) Structure and function of chitin-binding proteins. *Annu. Rev. Plant Physiol. Plant Mol. Biol.* **44**, 591–615.

Raper, C.A. (1988) *Schizophyllum commune*, a model for genetic studies of the basidiomycetes. In: *Genetics of Plant Pathogenic Fungi* (G.S. Sidhu, ed.), pp. 511–522. Academic Press, London.

Raper, J.R. (1966) *Genetics of Sexuality in Higher Fungi*. The Ronald Press Co, New York.

Raudaskoski, M. and Viitanen, H. (1982) Effects of aeration and light on fruit-body induction in *Schizophyllum commune*. *Trans. Brit. Mycol. Soc.* **78**, 89–96.

Rayner, A.D.M., Griffith, G.S. and Ainsworth, A.M. (1995) Mycelial interconnectedness. In: *The Growing Fungus* (N.A.R. Gow and M. Gadd, eds), pp. 21–40. Chapman and Hall, London.

Read, D.J. (1991) Mycorrhizas in ecosystems – nature's response to the "law of the minimum". In: *Frontiers in Mycology* (D.L. Hawksworth, ed.), pp. 101–130. CAB International, Wallingford.

Richards, W.C. (1993) Cerato-ulmin: a unique wilt toxin of instrumental significance in the development of Dutch elm disease. In: *Dutch Elm Disease Research, Cellular and*

Molecular Approaches (M.B. Sticklen and J.L. Sherald, eds), pp. 89–151. Springer Verlag, New York.

Rohe, M., Gierlich, A., Hermann, H., Hahn, M., Schmidt, B., Rosahl, S. and Knogge, W. (1995) The race-specific elicitor, NIP1, from the barley pathogen, *Rhynchosporium secalis*, determines avirulence on host plants of the Rrs1 resistance genotype. *EMBO J.* **14**, 4168–4177.

Rosenberg, M. and Kjelleberg, S. (1986) Hydrophobic interactions: role in bacterial adhesion. *Adv. Microb. Ecol.* **9**, 353–393.

Rotenberg, Y., Boruvka, L. and Neumann, A.W. (1983) Determination of surface tension and contact angle from the shapes of axisymmetric fluid interfaces. *J. Colloid Interface Sci.* **93**, 169–183.

Rouxhet, P.G., Mozes, N., Dengis, P.B., Dufrene, Y.F., Gerin, P.A. and Genet, M.J. (1994) Application of X-ray photoelectron spectroscopy to microorganisms. *Colloids Surf. B* **2**, 347–369.

Ruiters, M.H.J. and Wessels, J.G.H. (1989) *In situ* localization of specific RNAs in whole fruiting colonies of *Schizophyllum commune*. *J. Gen. Microbiol.* **135**, 1747–1754.

Ruiters, M.H.J., Sietsma, J.H. and Wessels, J.G.H. (1988) Expression of dikaryon-specific mRNAs of *Schizophyllum commune* in relation to incompatibility genes, light and fruiting. *Exp. Mycol.* **12**, 60–69.

Russo, P.S., Blum, F.D., Ipsen, J.D., Abul-Hajj, Y.J. and Miller, W.G. (1982) The surface activity of the phytotoxin cerato-ulmin. *Can. J. Bot.* **60**, 1414–1422.

Sassen, M.M.A., Remsen, C.C. and Hess, W.M. (1967) Fine structure of *Penicillium megasporum* conidiospores. *Protoplasma* **64**, 75–87.

Schuren, F.H.J. and Wessels, J.G.H. (1990) Two genes specifically expressed in fruiting dikaryons of *Schizophyllum commune*: homologies with a gene not regulated by mating type genes. *Gene* **90**, 199–205.

Schuren, F.H.J., Ásgeirsdóttir, S.A., Kothe, E.M., Scheer, J.H.J. and Wessels, J.G.H. (1993a) The *Sc7/Sc14* gene family of *Schizophyllum commune* codes for extracellular proteins specifically expressed during fruit-body formation. *J. Gen. Microbiol.* **139**, 2083–2090.

Schuren, F.H.J., Harmsen, M.C. and Wessels, J.G.H. (1993b) A homologous gene-reporter system for the basidiomycete *Schizophyllum commune* based on internally deleted genes. *Mol. Gen. Genet.* **238**, 91–96.

Schuren, F.H.J., van der Lende, T.R. and Wessels, J.G.H. (1993c) Fruiting genes of *Schizophyllum commune* are transcriptionally regulated. *Mycol. Res.* **97**, 538–542.

Selitrennikoff, C.P. (1976) *Easily wettable*, a new mutant. *Neurospora Newsl.* **23**, 23.

Service, R.F. (1994) Self-assembly comes together. *Nature* **265**, 316–318.

Smucker, R.A. and Pfister, R.M. (1978) Characterization of *Streptomyces coelicolor* A3(2) aerial spore mozaic. *J. Gen. Microbiol.* **24**, 397–408.

Sokolovsky, V.Y., Lauter, F.-R., Müller-Röber, B., Ricci, M., Schmidhauser, T.J. and Russo, V.E.A. (1992) Nitrogen regulation of blue light-inducible genes in *Neurospora crassa*. *J. Gen. Microbiol.* **138**, 2045–2049.

Springer, J. and Wessels, J.G.H. (1989) A frequently occurring mutation that blocks the expression of fruiting genes in *Schizophyllum commune*. *Mol. Gen. Genet.* **219**, 486–488.

St Leger, R.J., Staples, R.C. and Roberts, D.W. (1992) Cloning and regulatory analysis of starvation stress gene, *ssgA*, encoding a hydrophobin-like protein from the entomopathogenic fungus, *Metarhizium anisopliae*. *Gene* **120**, 119–124.

Sterk, P., Booij, H., Schellekens, G.A., Van Kammen, A. and de Vries, S.C. (1991) Cell-specific expression of the carrot lipid transfer protein gene. *Plant Cell* **3**, 907–921.

Stevenson, K.J., Slater, J.A. and Takai, S. (1979) Cerato-ulmin, a wilting toxin of Dutch elm disease fungus. *Phytochemistry* **18**, 235–238.

Sticklen, M.B. and Bolyard, M. (1994) Refinement of physiological roles for cerato-ulmin by analogy with other hydrophobins. *Trends Microbiol.* **2**, 213–215.

Stringer, M.A. and Timberlake, W.E. (1993) Cerato-ulmin, a toxin involved in Dutch elm disease, is a hydrophobin. *Plant Cell* **5**, 145–146.

Stringer, M.A. and Timberlake, W.E. (1995) *dewA* encodes a fungal hydrophobin component of the *Aspergillus* spore wall. *Mol. Microbiol.* **16**, 33–44.

Stringer, M.A., Dean, R.A., Sewall, T.C. and Timberlake, W.E. (1991) *Rodletless*, a new *Aspergillus* developmental mutant induced by directed gene inactivation. *Genes Dev.* **5**, 1161–1171.

Svircev, A.M., Jeng, R.S. and Hubbes, M. (1988) Detection of cerato ulmin on aggressive isolates of *Ophiostoma ulmi* by immunocytochemistry and scanning electron microscopy. *Phytopathology* **78**, 322–327.

Tagu, D., Nasse, B. and Martin, F. (1996) Cloning and characterization of hydrophobins encoding cDNAs from the ectomycorrhizal basidiomycete *Pisolithus tinctorius*. *Gene* **168**, 93–97.

Takai, S. (1974) Pathogenicity and cerato-ulmin production in *Ceratocystis ulmi*. *Nature* **252**, 124–126.

Takai, S. (1980) Relationship of the production of toxin, cerato-ulmin, to synnemata formation, pathogenicity, mycelial habit and growth of *Ceratocystis ulmi* isolates. *Can. J. Bot.* **58**, 658–662.

Takai, S. and Hiratsuka, Y. (1980) Accumulation of the material containing the toxin cerato-ulmin on the hyphal surface of *Ceratocystis ulmi*. *Can. J. Bot.* **58**, 658–662.

Takai, S. and Hiratsuka, Y. (1984) Scanning electron microscope observations of internal symptons of white elm following *Ceratocystis ulmi* infection and cerato-ulmin treatment. *Can. J. Bot.* **62**, 1365–1371.

Takai, S. and Richards, W.C. (1978) Cerato-ulmin, a wilting toxin of *Ceratocystis ulmi*: isolation and some properties of cerato-ulmin from the culture of *C. ulmi*. *Phytopathol. Z.* **91**, 129–146.

Talbot, N.J., Ebbole, D.J. and Hamer J.E. (1993) Identification and characterization of *MPG1*, a gene involved in pathogenicity from the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **5**, 1575–1590.

Talbot, N.J., Kershaw, M., Wakley, G.E., de Vries, O.M.H., Wessels, J.G.H. and Hamer, J.E. (1996) *MPG1* encodes a fungal hydrophobin involved in surface interactions during infection-related development of the rice blast fungus *Magnaporthe grisea*. *Plant Cell* **8**, 985–999.

Templeton, M.D., Rikkerink, E.H.A. and Beever, R.E. (1994) Small, cysteine-rich proteins and recognition in fungal–plant interactions. *Mol. Plant–Microbe Interact.* **7**, 320–325.

Templeton, M.D., Greenwood, D.R. and Beever, R.E. (1995) Solubilization of *Neurospora crassa* rodlet proteins and identification of the predominant protein as the proteolytically processed *eas* (*ccg-2*) gene product. *Exp. Mycol.* **19**, 166–169.

Terhune, B.T. and Hoch, H.C. (1993) Substrate hydrophobicity and adhesion of *Uromyces* urediospores and germlings. *Exp. Mycol.* **17**, 241–251.

Terras, F.R.G., Eggermont, K., Kovaleva, V., Raikhel, N.V., Osborn R.W., Kester, A., Rees, S.B., Torrekens, S., Van Leuven, F., Vanderleyden, J., Cammue, B.P.A. and Broekaert, W.F. (1995) Small cysteine-rich antifungal proteins from radish: their role in host defence. *Plant Cell* **7**, 573–588.

Thau, N., Monod, M., Crestani, B., Rolland, C., Tronchin, G., Latgé, J.-P. and Paris, S. (1994) *rodletless* mutants of *Aspergillus fumigatus*. *Inf. Immun.* **62**, 4380–4388.

Thomas, D. (1995) Nanotechnology's many disciplines. *Biotechnology* **13**, 439–443.

Timberlake, W.E. (1990) Molecular genetics of *Aspergillus* development. *Annu. Rev. Genet.* **24**, 5–36.

Timberlake, W.E. (1993) Translational triggering and feedback fixation in the control of fungal development. *Plant Cell* **5**, 1453–1460.

Trinci, A.P.J. (1974) A study of the kinetics of hyphal extension and branch initation of fungal mycelia. *J. Gen. Microbiol.* **81**, 225–236.

Trinci, A.P.J., Wiebe, M.G. and Robson, G.D. (1994) The mycelium as an integrated entity. In: *The Mycota*, Vol. I, *Growth, Differentiation and Development* (J.G.H. Wessels and F. Meinhardt, eds), pp. 175–193. Springer Verlag, Berlin.

Troughton, J.H. and Hall, D.M. (1967) Extracuticular wax and contact angle measurements on wheat (*Triticum vulgare* L.) *Aust. J. biol. Sci.* **20**, 509–525.

van den Ackerveken, G.F.J.M., van Kan, J.A.L., Joosten, M.H.A.J., Muisers, J.M., Verbakel, H.M. and de Wit, P.J.G.M. (1993) Characterization of two putative pathogenicity genes of the fungal pathogen *Cladosporium fulvum*. *Mol. Plant–Microbe Interact.* **6**, 210–215.

van der Valk, P. and Marchant, R. (1978) Hyphal ultrastructure in fruit-body primordia of the basidiomycetes *Schizophyllum commune* and *Coprinus cinereus*. *Protoplasma* **95**, 57–72.

van der Vegt, W., van der Mei, H.C., Wösten, H.A.B., Wessels, J.G.H. and Busscher, H.J. (1996) A comparison of the surface activity of the fungal hydrophobin SC3p with those of other proteins. *Biophys. Chem.* **51**, 253–260.

van Wetter, M.-A., Schuren, F.H.J., Schuurs, T.A. and Wessels, J.G.H. (1996) Targeted mutation of the *SC3* hydrophobin gene of *Schizophyllum commune* affects formation of aerial hyphae. *FEMS Microbiol. Lett.* (in press).

Wendland, J., Vaillancourt, L.J., Hegner, J., Lengeler, K.B., Laddison, K.J., Specht, C.A., Raper, C.A. and Kothe, E. (1995) The mating type locus *Bα1* of *Schizophyllum commune* contains a pheromone receptor and putative pheromone genes. *EMBO J.* **14**, 5271–5278.

Wessels, J.G.H. (1978) Incompatibility factors and the control of biochemical processes. In: *Genetics and Morphogenesis in the Basidiomycetes* (M.N. Schwalb and P.G. Miles, eds), pp. 81–104. Academic Press, New York.

Wessels, J.G.H. (1986) Cell wall synthesis in apical hyphal growth. *Int. Rev. Cytol.* **104**, 37–79.

Wessels, J.G.H. (1990) Role of cell wall architecture in fungal tip growth generation. In: *Tip Growth of Plant and Fungal Cells* (I.B. Heath, ed.), pp. 1–29. Academic Press, San Diego.

Wessels, J.G.H. (1992) Gene expression during fruiting in *Schizophyllum commune*. *Mycol. Res.* **96**, 609–620.

Wessels, J.G.H. (1993a) Wall growth, protein excretion and morphogenesis in fungi. *New Phytol.* **123**, 397–413.

Wessels, J.G.H. (1993b) Fruiting in the higher fungi. *Adv. Microb. Physiol.* **34**, 147–202.

Wessels, J.G.H. (1994) Developmental regulation of fungal cell wall formation. *Annu. Rev. Phytopathol.* **32**, 413–437.

Wessels, J.G.H. (1996) Fungal hydrophobins: proteins that function at an interface. *Trends Plant Sci.* **1**, 9–15.

Wessels, J.G.H. and Sietsma, J.H. (1979) Wall structure and growth in *Schizophyllum commune*. In: *Fungal Walls and Hyphal Growth* (J.H. Burnett and A.P.J. Trinci, eds), pp. 27–48. Cambridge University Press, Cambridge.

Wessels, J.G.H. and Sietsma, J.H. (1981) Fungal walls: a survey. In: *Encyclopedia of Plant Physiology*, Vol. 13B (W. Tanner and F.A. Loewus, eds), pp. 352–394. Springer Verlag, Berlin.

Wessels, J.G.H., Kreger, D.R., Marchant, R., Regensburg, B.A. and de Vries, O.M.H. (1972) Chemical and morphological characterization of the hyphal wall surface of the basidiomycete *Schizophyllum commune*. *Biochim. Biophys. Acta* **273**, 346–358.

Wessels, J.G.H., Mulder, G.H. and Springer, J. (1987) Expression of dikaryon-specific and

non-specific mRNAs of *Schizophyllum commune* in relation to environmental conditions and fruiting. *J. Gen. Microbiol.* **133**, 2557–2561.

Wessels, J.G.H., de Vries, O.M.H., Ásgeirsdóttir, S.A. and Schuren, F.H.J. (1991a) Hydrophobin genes involved in formation of aerial hyphae and fruit bodies in Schizophyllum. *Plant Cell* **3**, 793–799.

Wessels, J.G.H, de Vries, O.M.H., Ásgeirsdóttir, S.A. and Springer, J. (1991b) The *thn* mutation of *Schizophyllum commune*, which suppresses formation of aerial hyphae, affects expression of the *SC3* hydrophobin gene. *J. Gen. Microbiol.* **137**, 2439–2445.

Wessels, J.G.H., Ásgeirsdóttir, S.A., Birkenkamp K.U., de Vries, O.M.H., Lugones, L.G., Schuren, F.H.J., Schuurs, T.A., van Wetter, M.A. and Wösten, H.A.B. (1995) Genetic regulation of emergent growth in *Schizophyllum commune*. *Can. J. Bot.* **73** (Suppl. 1), S273–S281.

Wildermuth, H., Wehrli, E. and Horne, R.W. (1971) The surface structure of spores and aerial mycelium in *Streptomyces coelicolor*. *J. Ultrastruct. Res.* **35**, 168–180.

Williams, S.J. Bradshaw, R.M., Costerton, J.W. and Forge, A. (1972) Fine structure of the spore sheath of some Streptomyces species. *J. Gen. Microbiol.* **72**, 249–258.

Wnendt, S., Ulbrich, N. and Stahl, U. (1994) Molecular cloning, sequence analysis and expression of the gene encoding an antifungal-protein from *Aspergillus giganteus*. *Curr. Genet.* **25**, 519–523.

Wösten H.A.B., Moukha, S.M., Sietsma, J.H. and Wessels J.G.H. (1991) Localization of growth and secretion of proteins in *Aspergillus niger*. *J. Gen. Microbiol.* **137**, 2017–2023.

Wösten, H.A.B., de Vries, O.M.H. and Wessels, J.G.H. (1993) Interfacial self-assembly of a fungal hydrophobin into a hydrophobic rodlet layer. *Plant Cell* **5**, 1567–1574.

Wösten, H.A.B., Schuren F.H.J. and Wessels, J.G.H. (1994a) Interfacial self-assembly of a hydrophobin into an amphipathic membrane mediates fungal attachment to hydrophobic surfaces. *EMBO J.* **13**, 5848–5854.

Wösten, H.A.B., Ásgeirsdóttir, S.A., Krook J.H., Drenth J.H.H. and Wessels, J.G.H. (1994b) The fungal hydrophobin SC3p self-assembles at the surface of aerial hyphae as a protein membrane constituting the hydrophobic rodlet layer. *Eur. J. Cell Biol.* **63**, 122–129.

Wösten, H.A.B., de Vries, O.M.H., van der Mei, H.C., Busscher, H.J. and Wessels, J.G.H. (1994c) Atomic composition of the hydrophobic and hydrophilic sides of self-assembled SC3p hydrophobin. *J. Bacteriol.* **176**, 7085–7086.

Wösten, H.A.B., Ruardy, T.G., van der Mei, H.C., Busscher, H.J. and Wessels, J.G.H. (1995) Interfacial self-assembly of a *Schizophyllum commune* hydrophobin into an insoluble amphipathic membrane depends on surface hydrophobicity. *Colloids Surf. B: Biointerfaces* **5**, 189–195.

Xiao, J.-Z., Ohshima, A., Kamakura, T., Ishiama, T. and Yamaguchi, I. (1994) Extracellular glycoprotein(s) associated with cellular differentiation in *Magnaporthe grisea*. *Mol. Plant–Microbe Interact.* **7**, 639–644.

Yaguchi, M., Pusztai-Carey, M., Roy, C., Surewicz, W.K., Carey, P.R., Stevenson, K.J., Richards W.C. and Takai, S. (1993) Amino-acid sequence and spectroscopic studies of Dutch elm disease toxin, cerato-ulmin. In: *Dutch Elm Disease Research, Cellular and Molecular Approaches* (M.B. Sticklen and J.L. Sherald, eds), pp. 152–170. Springer Verlag, New York.

Yli-Mattila, T., Ruiters, M.H.J., Wessels, J.G.H. and Raudaskoski, M. (1989a) Effect of inbreeding and light on monokaryotic and dikaryotic fruiting in the homobasidiomycete *Schizophyllum commune*. *Mycol. Res.* **93**, 535–542.

Yli-Mattila, T., Ruiters, M.H.J. and Wessels, J.G.H. (1989b) Photoregulation of dikaryon-specific mRNAs and proteins by UV-A light in *Schizophyllum commune*. *Curr. Microbiol.* **18**, 289–295.

Zentmeyer, G.A. (1942) Toxin formation by *Cerastomella ulmi*. *Science* **95**, 512–513.

Zhang, L., Villalon, D., Sun, Y., Kazmierczak, P. and van Alfen, N.K. (1994) Virus-associated down-regulation of the gene encoding cryparin, an abundant cell-surface protein from the chestnut blight fungus *Cryphonectria parasitica*. *Gene* **139**, 59–64.