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REVIEW

Hydrophobins and Repellents: Proteins with Fundamental Roles in Fungal Morphogenesis

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Kershaw, M. J., Talbot, N. J. 1998. Hydrophobins and Repellents: Proteins with Fundamental Roles in Fungal Morphogenesis. *Fungal Genetics and Biology* **23,** 18–33. Fungal hydrophobins are secreted proteins which react to interfaces between fungal cell walls and the air or between fungal cell walls and solid surfaces. They have been shown to be important in many morphogenetic processes, including sporulation, fruit body development, and infection structure formation. Hydrophobins form hydrophobic surface layers by self-assembly of secreted protein monomers in response to the environment. This process results in amphipathic polymers of interwoven rodlets on surfaces of fungal aerial structures and hyphal aggregations. Hydrophobin self-assembly is also involved in attachment of hyphae to hydrophobic surfaces and this may act as a conformational cue for certain developmental processes. Although hydrophobins appear to be ubiquitous among fungal taxa, a second class of fungal protein with very different biochemical characteristics could fulfill a similar role. These proteins, called repellents, have been identified in only one fungal species so far, but clearly help to make aerial hyphae hydrophobic. The functional similarities between hydrophobins and repellents highlight the importance of aerial development to the fungal lifestyle. © 1998 Academic Press

[1] Abbreviations used: TFA, trifluoroacetic acid; BRE, bristle response elements.

Index Descriptors: fungal development; conidiation; mushroom development; aerial morphogenesis; cysteine-rich proteins; bimodular proteins; mating-type genes; clock-control genes; filament formation.

To conquer terrestrial ecosystems, fungi have developed ways of spreading propagules to new locations and a capacity to withstand the inevitable desiccation which occurs on land. To do this they evolved the ability to produce upwardly projecting aerial hyphae and fruiting structures. These allow spores to be spread to new environments and also insulate the underlying mycelium from water loss. Evolution of aerial structures was therefore likely to have been important for the colonization of land by fungi and consequently to the success of the Mycota. During the past 5 years it has become clear that aerial morphogenesis involves fungal hydrophobins, secreted proteins which respond to the prevailing external environment (Wessels, 1996). Hydrophobins react to interfaces between solids and liquids or between liquids and the air. Recently, however, a further group of proteins called repellents has been found which could fulfill a similar function, but by a different mechanism (Wösten *et al.,* 1996). The discovery of these two unusual classes of proteins indicates that the production of hydrophobic aerial hyphae is such an important trait that it may occur via a number of distinct routes. The fungal hydrophobins have recently been reviewed by Wessels (1997) and the reader is referred to this work for a comprehensive

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treatment of the isolation, purification, and functional characterization of hydrophobins. In this review we describe the properties of fungal hydrophobins and repellents and discuss their likely roles in fungal development. We also speculate about the origins of these remarkable proteins and suggest investigations which might clarify their functions.

GENERAL PROPERTIES OF FUNGAL HYDROPHOBINS

Hydrophobins are widely found among the fungal taxa and have now been reported in 20 species, including members of the ascomycetes, basidiomycetes, and zygomycetes (de Vries *et al.,* 1993; Wessels, 1996). Hydrophobins are therefore likely to be ubiquitous in filamentous fungi (Wessels, 1996, 1997) and appear to have diverse functions (see Table 1). They are normally abundant proteins and are found in aerial hyphae, on the surface of fungal spores and infection structures, or on the surface of fruiting bodies. Hydrophobins have escaped detection until recently, however, because of their unusual biochemical properties and in almost all cases they were discovered by identifying the corresponding gene.

Fungal hydrophobins are small, secreted, moderately hydrophobic proteins with eight cysteine residues ordered in a particular manner in their amino acid sequence (see Table 2). Hydrophobin genes all contain signal peptide sequences and secretion has been confirmed by immunolocalization of two hydrophobins (Wösten *et al.,* 1994a; Lugones *et al.,* 1996) and by protein purification (Talbot *et al.,* 1996; Lora *et al.,* 1995) and N-terminal sequence analysis for a number of others (Templeton *et al.,* 1995; Bidochka *et al.,* 1995). The spacing of cysteine residues varies, but is based on a general configuration shown in Table 2. The cysteine spacing shows considerable variation in the Class I hydrophobins but is invariant in the Class II hydrophobins and all cysteine residues appear to be involved in disulfide bridge formation. This is based on studies of the Sc3 hydrophobin that showed no free sulfhydryl groups (de Vries *et al.,* 1993) and on biochemical analysis of the Class II hydrophobin cerato-ulmin, which determined disulfide linkages between all eight cysteine residues (Yaguchi *et al.,* 1993). The cysteine linkages identified in cerato-ulmin were between Cys_1 and Cys_2, Cys_3 and Cys_4, Cys_5 and Cys_6, and Cys_7 and Cys_8. This predicts a protein with four "loops," two of which are composed predominantly of hydrophobic residues (for a schematic representation of a possible generic hydrophobin see Fig. 1). The characteristic order of the eight cysteine residues defines the fungal hydrophobins and distinguishes them from other cysteine-rich proteins such as peptide elicitors (Van den Ackervecken *et al.,* 1992; Templeton *et al.,* 1994; Rohe *et al.,* 1995), chitin-binding proteins, lipid-binding proteins (Sterk *et al.,* 1991; Désormeaux *et al.,* 1992; Castonguay *et al.,* 1994), and extracellular toxins. There are, however, a number of similar features among these secreted disulfide-rich plant and fungal proteins (Templeton *et al.,* 1994; Lora *et al.,* 1995). They are all, for example, small proteins with a high percentage of hydrophobic residues leading to a general hydrophobic configuration. It is interesting, however, that while cystallographic data are available for many of the plant disulfide-rich secreted proteins (crambin, HPS, and lipid-binding proteins), very few have had their biological functions determined unequivocally (Teeter and Whitlow, 1986; José-Estanyol *et al.,* 1992; Désormeaux *et al.,* 1992; Castonguay *et al.,* 1994). In contrast, studies on hydrophobins have often determined biological function—at least in outline—but structural information has been hard to determine (Templeton *et al.,* 1995). Comparative analysis between these plant and fungal proteins may therefore be useful in defining the structure–function relationships for both classes (Lora *et al.,* 1995). A clear example of this is the hydrophobic secreted protein from soybean, HPS, which shows a compact structure linked by disulfide bridges, with hydrogen bonds stabilizing intramolecular interactions (Baud *et al.,* 1993). This protein adopts a structure somewhat similar to that which might be predicted for a hydrophobin monomer and, importantly, in plant cell wall extracts HPS is often found as part of a larger protein assembly—perhaps suggesting properties similar to those of hydrophobins.

Despite an apparent structural similarity, the amino acid homology between hydrophobins is limited. The identity between the products of the *rodA, MPG1, EAS, Sc3, Sc4, Sc1, ssgA,* and *C-U* genes, for example, is only 11% at the amino acid level and even among the *S. commune* hydrophobins it is only 40%. The amino acid similarity of 20 hydrophobins was recently compared by Wessels (1997), after removal of signal peptides at positions determined by either biochemical analysis or consensus sequence comparison. He observed 4.3% amino acid identity and only 1.7% similarity among all identified hydrophobins. This considerable divergence is surprising and may represent either diversity in hydrophobin function—suggested by the patterns of their expression in a variety of developmental processes—or that significant redundancy in amino acid usage can occur without loss of general hydrophobin function.

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TABLE 1
The Fungal Hydrophobins

Name	Hydrophobin class	Taxonomic class	Organism	Mutant phenotype determined	Biological function	Reference
SC1	I	Basidiomycetes	*Schizophyllum commune*	−	Unknown. Expressed in dikaryotic phase.	Schuren and Wessels (1990); Wessels *et al.* (1991)
SC3	I			+	Involved in aerial hyphae formation and ability to attach to hydrophobic surfaces.	Wösten *et al.* (1994) Van Wetter *et al.* (1996)
SC4	I			−	Unknown. Expressed in dikaryotic phase. Known to line gas channels in basidiome.	Schuren and Wessels (1990); Wessels *et al.* (1991)
SC6	I			−	Unknown. Expressed in dikaryotic phase.	Wessels (1997)
CoH1	I		*Coprinus cinereus*	−	Unknown.	Asgeirsdottir *et al.* (1997)
HydPt-1	I		*Pisolithus tinctorius*	−	Unknown. Expressed in mycorrhiza.	Tagu *et al.* (1996)
HydPt-2	I			−	Unknown. Expressed in mycorrhiza.	Tagu *et al.* (1996)
ABH-1	I		*Agaricus bisporus*	−	Unknown. Expressed in dikaryotic phase. Known to line gas channels in basidiome.	Lugones *et al.* (1996); de Groot *et al.* (1996)
ABH-2	I			−	Unknown. Expressed in dikaryotic phase.	Lugones *et al.* (1996); de Groot *et al.* (1996)
SSGA	I	Ascomycetes	*Metarhizium anisopliae*	−	Unknown. Expressed during appressorium development.	St. Leger *et al.* (1992)
MPG1	I		*Magnaporthe grisea*	+	Conidial spore wall protein. Involved in conidium and appressorium formation. Required for full pathogenicity.	Talbot *et al.* (1993) Talbot *et al.* (1996)
RodA	I		*Aspergillus nidulans*	+	Conidial spore wall protein.	Stringer *et al.* (1991)
DewA	I		*Aspergillus nidulans*	+	Conidial spore wall protein.	Stringer and Timberlake (1995)
HYP1	I		*Aspergillus fumigatus*	+	Conidial spore wall protein.	Parta *et al.* (1994) Thau *et al.* (1994)
Eas	I		*Neurospora crassa*	+	Conidial spore wall protein.	Bell-Pederson *et al.* (1992) Lauter *et al.* (1992)
CU	II		*Ophiostoma ulmi*	+	Aerial hyphae formation and hydrophobicity. Abundant expression in host plant.	Bowden *et al.* (1996)
CRYP	II		*Cryphonectria parasitica*	+	Unknown. Abundant expression in host plant. A cell wall protein.	Zhang *et al.* (1994)
QID3	II		*Trichoderma harzianum*	−	Unknown. Highly expressed in chitin-containing medium.	Lora *et al.* (1995)
HFB1	II		*Trichoderma reesei*	−	Unknown. Highly expressed in glucose-containing medium.	Nakari-Setälä *et al.* (1996)
HFB2	II		*Trichoderma reesei*	−	Unknown.	Nakari-Setälä and Pentilla (unpublished)
cpa3	II		*Claviceps purpurea*	−	Unknown. Unusual “tri-hydrophobin” structure. Expressed in alkaloid-producing cultures.	Arntz and Tudzynski (1997)

CLASS I AND CLASS II HYDROPHOBINS

A significant difference in the arrangement of amino acid residues is evident among the hydrophobins. This is apparent when hydropathy plots are produced after alignment of the cysteine residues within the hydrophobin sequence and was used by Wessels (1994) to resolve the hydrophobins into two groups which he designated Class I and Class II (Table 2). An example of a putative basic structure for each type of hydrophobin is given in Fig. 1. The diagrams assume a basic organization for hydrophobins around four disulfide bridges. While this is highly speculative—because the presence of these intramolecular

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TABLE 2
Cysteine Spacing within the Fungal Hydrophobins

Name of hydrophobin	Cysteine spacing[a]	Diameter of rodlets[b]
Consensus (Class I):	$C\text{-}X_{5-7}C\text{-}C\text{-}X_{19-39}\text{-}C\text{-}X_{8-23}\text{-}C\text{-}X_{5}\text{-}C\text{-}C\text{-}X_{6-18}\text{-}C\text{-}X_{2-13}$	
MPG1	$C\text{-}X_7C\text{-}C\text{-}X_{22}\text{-}C\text{-}X_{19}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_5$	5–7 nm
rodA	$C\text{-}X_7C\text{-}C\text{-}X_{39}\text{-}C\text{-}X_{18}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{17}\text{-}C\text{-}X_7$	10–12 nm
dewA	$C\text{-}X_6C\text{-}C\text{-}X_{30}\text{-}C\text{-}X_{23}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_6\text{-}C\text{-}X_{13}$	?
Sc3	$C\text{-}X_6C\text{-}C\text{-}X_{33}\text{-}C\text{-}X_{12}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_6$	10–13 nm
ssgA	$C\text{-}X_5C\text{-}C\text{-}X_{19}\text{-}C\text{-}X_{15}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_5$	?
Sc1	$C\text{-}X_6C\text{-}C\text{-}X_{33}\text{-}C\text{-}X_{12}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_7$	?
Sc4	$C\text{-}X_6C\text{-}C\text{-}X_{33}\text{-}C\text{-}X_{12}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_5$	?
Eas	$C\text{-}X_8C\text{-}C\text{-}X_{25}\text{-}C\text{-}X_8\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{18}\text{-}C\text{-}X_2$	10 nm
HydPt-1	$C\text{-}X_6C\text{-}C\text{-}X_{32}\text{-}C\text{-}X_{13}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_7$	?
HydPt-2	$C\text{-}X_6C\text{-}C\text{-}X_{32}\text{-}C\text{-}X_{13}\text{-}C\text{-}X_5\text{-}C\text{-}C\text{-}X_{12}\text{-}C\text{-}X_7$	?
Consensus (Class II):	$C\text{-}X_{9-10}C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_{8-9}\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}X_{6-7}$	
Cerato-ulmin	$C\text{-}X_9C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_9\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}X_6$	?
Cryparin	$C\text{-}X_9C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_8\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}X_6$	?
QID3	$C\text{-}X_9C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_9\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}K_7$[c]	?
HFB1	$C\text{-}X_9C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_8\text{-}C\text{-}C\text{-}X_{10}\text{-}C\text{-}X_6$	?
cpa3	$C\text{-}X_{9-10}C\text{-}C\text{-}X_{11}\text{-}C\text{-}X_{16}\text{-}C\text{-}X_8\text{-}C\text{-}C\text{-}X_{10}\text{-}C$[d]	?

[a] (C, cysteine; X, any amino acid). The number of amino acid residues preceding the first cysteine cannot be accurately determined because signal peptide cleavage sites have not been determined in all cases.

[b] Where determined by ultrastructural analysis.

[c] The published QID3 sequence suggests that the second cysteine residue may be a serine (Lora *et al.*, 1995).

[d] Spacing based on incomplete sequence data from multiple hydrophobin domains of cpa3 (Arntz and Tudzynski, 1997).

disulfide linkages has been proven only for cerato-ulmin (Yaguchi *et al.*, 1993)—it does illustrate how a general hydrophobin structure might be organized. Clear differences between Class I and Class II hydrophobins can be seen, for example, in the grouping of hydrophobic and hydrophilic residues along the primary sequence and the cysteine spacing is also diagnostic for each group. The Class I hydrophobins, for example, potentially constitute two-domain proteins with four loops centered around the disulfide linkages. The second and fourth loops vary greatly in length between each hydrophobin, as shown in Figs. 1a and 1b. The Sc3 hydrophobin has a much larger second loop than MPG1p and this is also true for many of the other Class I hydrophobins, including the predicted products of *rodA* and *dewA* from *Aspergillus nidulans* (Table 2). In comparison the Class II hydrophobins putatively contain loops which are more invariant in length and the spacing between each domain is also invariant. In both cases the second and fourth loops are predominantly hydrophobic, containing bulky hydrophobic residues (M, I, L, V) surrounded by nonpolar amino acids (G, A) or residues not reducing the net hydropathy (T, Y). The hydrophobin monomers may therefore fold directly into an amphipathic configuration, although it must be recognized that the loops illustrated will themselves contain other secondary structure.

The differences in hydrophobin structure are consistent with biochemical investigations which have shown that Class I and Class II hydrophobins are distinct. Experiments with *Schizophyllum commune* showed that Class I hydrophobins could be purified from fungal cell walls as SDS-insoluble high-molecular-weight complexes (Wessels *et al.*, 1991a). These complexes can be dissociated into their constituent monomers by treatment with formic acid or with oxidizing agents such as performic acid. The latter treatment oxidizes cystine to cysteic acid, breaking disulfide bridges, and thereby permanently breaks the hydrophobin structure during extraction. Using this method hydrophobins were purified from *S. commune* and shown to correspond to the products of genes already cloned during differential screens to study dikaryotic gene expression (Mulder and Wessels, 1996; Wessels *et al.*, 1991a). Subsequently, however, it was found that ice-cold trifluoroacetic acid (TFA),[1] which disrupts short-range hydrophobic interactions, was very effective in dissociating hydrophobin assemblages, leaving the monomers intact and biologically active (de Vries *et al.*, 1993). Using this procedure Wessels and co-workers showed the widespread nature of hydrophobins among fungi (de Vries *et al.*, 1993) and discovered the process of interfacial self-assembly (Wösten *et al.*, 1993). This process is described in detail below but essentially defines the ability of extracted hydrophobin monomers to reassemble into amphipathic polymers after aeration of protein solutions.

The Class II hydrophobins by comparison had previously been studied in much greater detail, largely due to the pioneering work of Takai and co-workers on the phytotoxin cerato-ulmin from *Ophiostoma ulmi* (Richards and Takai, 1993; Takai, 1974, 1980). Only after the amino acid sequence of cerato-ulmin was derived (Bowden *et al.*, 1993) was it found to be homologous to that of hydrophobins (Stringer and Timberlake, 1993), although the purification of cerato-ulmin revealed a number of characteristics which strongly resembled those of the *S. commune* hydrophobins, including the ability of cerato-ulmin to polymerize into fibrils or rods when mixed with air. These aggregates were very unstable, however, compared to those described for the Sc3 hydrophobin and could be

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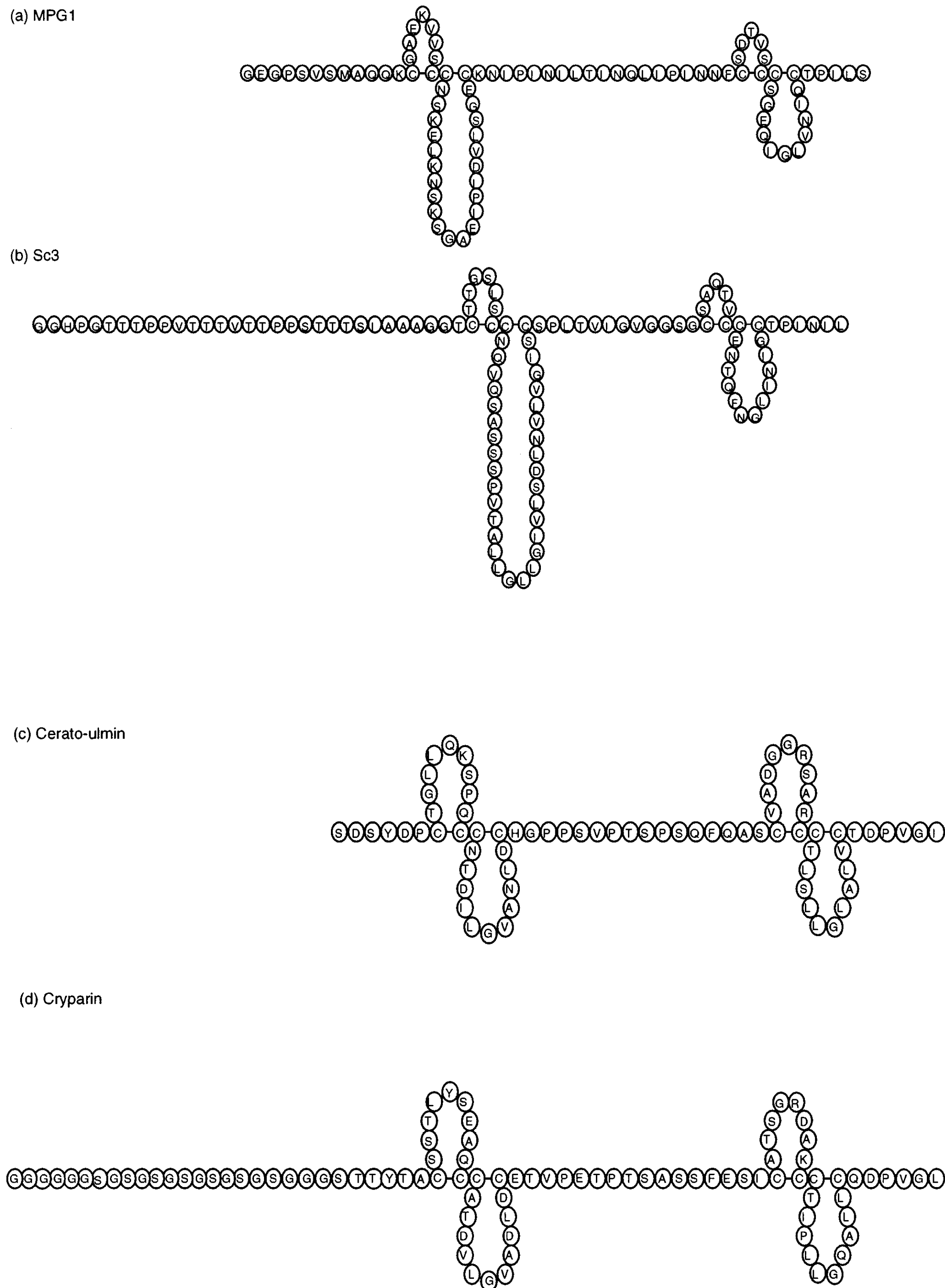

FIG. 1. Schematic representation of putative hydrophobin structures based on intramolecular disulfide linkages determined for cerato-ulmin (Yaguchi *et al.,* 1993). In this model the eight cysteine residues form four intramolecular disulfide bridges resulting in two-domain proteins containing four "loops." Loops 2 and 4 are predominantly hydrophobic. The Class I hydrophobins shown in (a) and (b) vary in the length of loops 1, 2, and 4, with loop 3 being invariant. The greatest divergence is in the length of loop 2, which varies from 22 to 39 amino acids. The distance between each domain also varies considerably. The examples shown are (a) MPG1 from *Magnaporthe grisea* and (b) Sc3 from *Schizophyllum commune.* The Class II hydrophobins show more conservation in spacing of cysteine residues leading to a broadly symmetrical structure with four loops of similar size, separated by an invariant number of amino acids. The examples shown are (c) cerato-ulmin from *Ophiostoma ulmi* and (d) cryparin from *Cryphonectria parasitica.* Note the long N-terminal glycine-rich tract in cryparin which is also found in the predicted product of *cpa3* from *Claviceps purpurea* (Arntz and Tudzynski, 1997). Cysteine residues, bulky hydrophobic residues (M, F, I, L, V), positively charged residues (K, R), and negatively charged residues (E, D) are shown. The representations are adapted from models originally constructed by Joseph Wessels.

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easily dissipated in 60% ethanol or by application of pressure (Takai and Richards, 1978).

INTERFACIAL SELF-ASSEMBLY

Interfacial self-assembly describes the ability of hydrophobins to respond to interfaces between water and air or between water and solid surfaces. Purification and characterization of Sc3 from *S. commune* lead to the discovery of self-assembly which appears to be accomplished by spontaneous aggregation of hydrophobin monomers mediated by short-range hydrophobic interactions (Wösten *et al.,* 1993). The aggregates are extremely insoluble and not dispersed even by extraction with hot detergents. Only by treating Sc3 with TFA, or formic acid, are the polymers dissociated into the component hydrophobin monomers (de Vries *et al.,* 1993).

Self-assembly of Sc3 immediately implicated hydrophobins in the development of aerial structures and this corresponds to their patterns of expression (Wessels *et al.,* 1991a). Studies with the Sc3 hydrophobin have also provided empirical evidence for this role (Wösten *et al.,* 1993). The Sc3 hydrophobin is secreted abundantly in submerged liquid cultures during monokaryotic growth of *S. commune.* This is in marked contrast to the remaining *S. commune* hydrophobins, which appear to function in the development of the dikaryotic basidiomes (Wessels, 1992). Self-assembly of Sc3 hydrophobin monomers was initially carried out using vigorous aeration of protein solutions but was also shown to occur at interfaces with water and mineral oil or between water and hydrophobic surfaces. The self-assembled hydrophobin was examined ultrastructurally and found to be composed of bundles of rodlets which appeared to be interwoven. Sc3 rodlets are 10–13 nm in diameter and present on the hydrophobic side of the polymerized hydrophobin layer (Wösten *et al.,* 1993, 1994a). The rodlets strongly resembled those found on the surfaces of fungal aerial hyphae and conidia described below (Hess *et al.,* 1968; Beever and Dempsey, 1978). Genetic evidence from *A. nidulans* (Stringer *et al.,* 1991) and immunolocalization of Sc3 firmly established that rodlet layers on fungal surfaces are the primary products of self-assembled hydrophobins (Wösten *et al.,* 1994a). The discovery of Sc3 self-assembly led to formulation of a model for fungal aerial morphogenesis which depends on secretion of hydrophobin monomers from hyphal tips (for reviews see Wessels, 1994, 1996, 1997). A representation of this model is shown in Fig. 2. Hydrophobin secretion occurs from hyphal tips growing in an aqueous environment. Upon encountering the interface with air, self-assembly occurs, which lowers the surface tension and allows the hypha to emerge into the air. As a hypha extends, continued secretion and self-assembly allow it to maintain its hydrophobic coating and grow into the air. Gene disruption of *Sc3* has supported this model, because *sc3*⁻ null mutants lack aerial hyphae (Van Wetter *et al.,* 1996) and can be complemented in *trans* using purified Sc3 protein (H. A. B. Wösten and J. G. H. Wessels, personal communication). The successful *trans*-complementation experiments support the view that Sc3 assemblages at air–water interfaces act as primary foci for the formation of new aerial hyphae. Consistent with this, Van der Vegt *et al.* (1996) showed that Sc3 is capable of lowering the surface tension of water from 72 to 32 mJ m^{-2}. This indicates that aerial development may be able to occur as long as this primary hydrophobin-mediated event can take place. The aerial hyphae resulting from the Sc3 *trans*-complementation experiments are, however, hydrophilic, which demonstrates that secretion of Sc3 from emerging hyphal tips is required for surface hydrophobicity.

The self-assembly process also has other important effects on fungal development. The *sc3*⁻ null mutants were, for example, shown to be unable to adhere to hydrophobic surfaces (Wösten *et al.,* 1994b). This highlighted the potential role of hydrophobins in surface interactions, suggested by studies with pathogenic fungi (Talbot *et al.,* 1993). Self-assembly on surfaces was found to be a function of the hydrophobicity of the surface. When hydrophobicity gradients were formed by treating glass with dichlorodimethylsilane, the self-assembly of Sc3 was shown to require a surface contact angle of between 60° and 90° (Wösten *et al.,* 1995). The self-assembly process may therefore be specifically adapted for hydrophobic surfaces and it will be important to determine whether this characteristic differs from one hydrophobin to another.

THE ROLE OF HYDROPHOBINS IN FUNGAL DEVELOPMENT

Structure and Properties of Rodlet Layers

In most cases hydrophobins were first identified as mRNAs abundantly transcribed during developmental processes such as sporulation, fruit body formation, or fungal infection of plants and insects. Genetic studies also implicated them in these morphogenetic processes and

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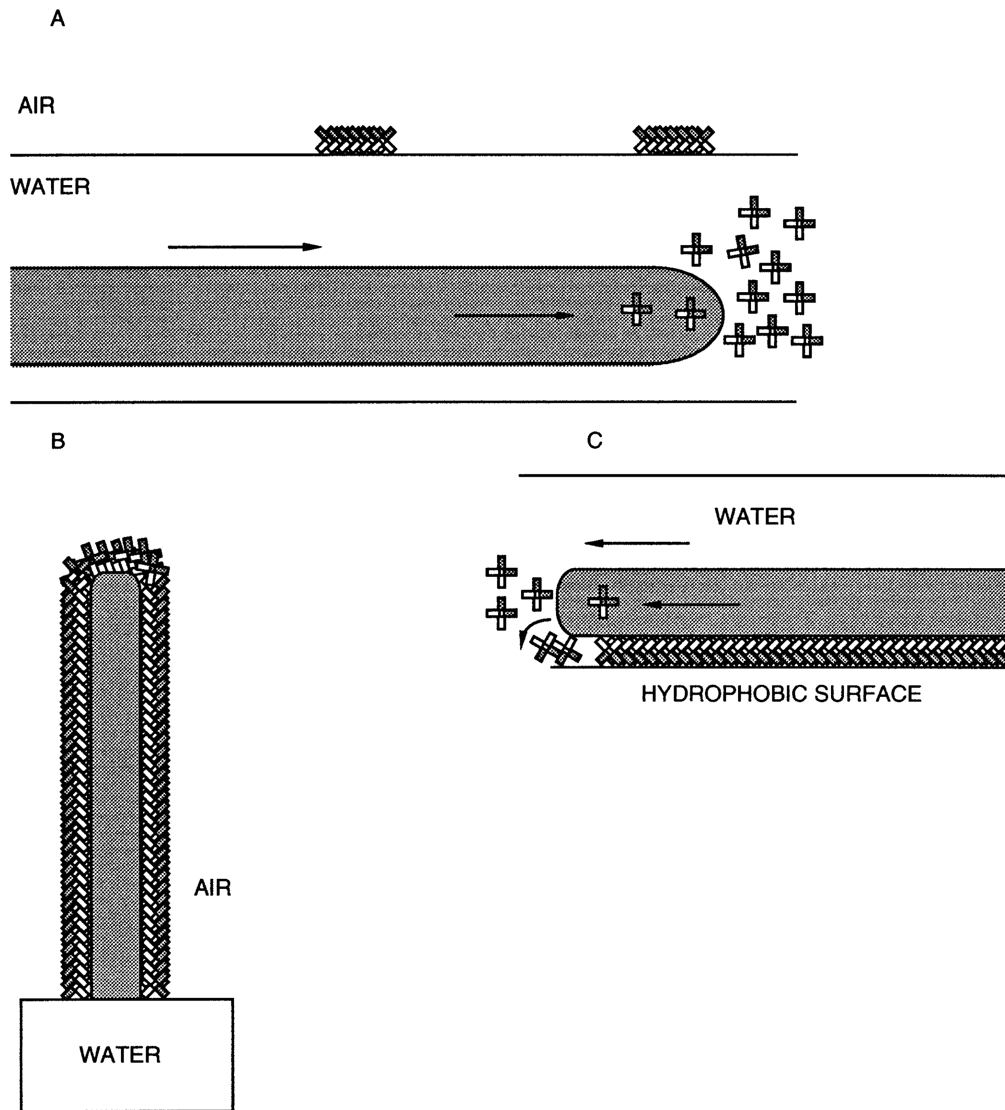

FIG. 2. Schematic representation of the hydrophobin self-assembly process. Hydrophobin monomers are shown with four external loops represented as the arms of a cross. The proteins fold such that one side is predominantly hydrophobic (shaded) and the other hydrophilic (white). The amphipathic hydrophobin monomers are secreted when the fungus is submerged (A) but self-assemble when the fungus reaches air–water interfaces (B) or hydrophobic surfaces (C). This figure is adapted from a model originally formulated by Wösten *et al.* (1994a) and is reproduced from Talbot (1997) from Current Biology Limited.

showed their role to be in the formation of rodlet protein layers found on fungal surfaces. Gene disruption of the *rodA* gene from *A. nidulans* and the *EAS* gene from *Neurospora crassa,* for example, produced mutants with spores which are "easily wetted," lacking the hydrophobic rodlet protein that normally coats the conidium (Stringer *et al.,* 1991; Bell-Pederson *et al.,* 1992; Lauter *et al.,* 1992). The rodlet layer of *N. crassa* has been characterized biochemically and shown to be composed of *EAS* hydrophobin (Beever and Dempsey, 1978; Dempsey and Beever, 1979; Templeton *et al.,* 1995). Hydrophobin-encoded rodlet layers are common features of aerial structures including mushroom caps, spores surfaces, and aerial hyphae themselves (see Fig. 3).

The role of rodlet layers in fungi is unclear. They are extremely hydrophobic and water contact angles with

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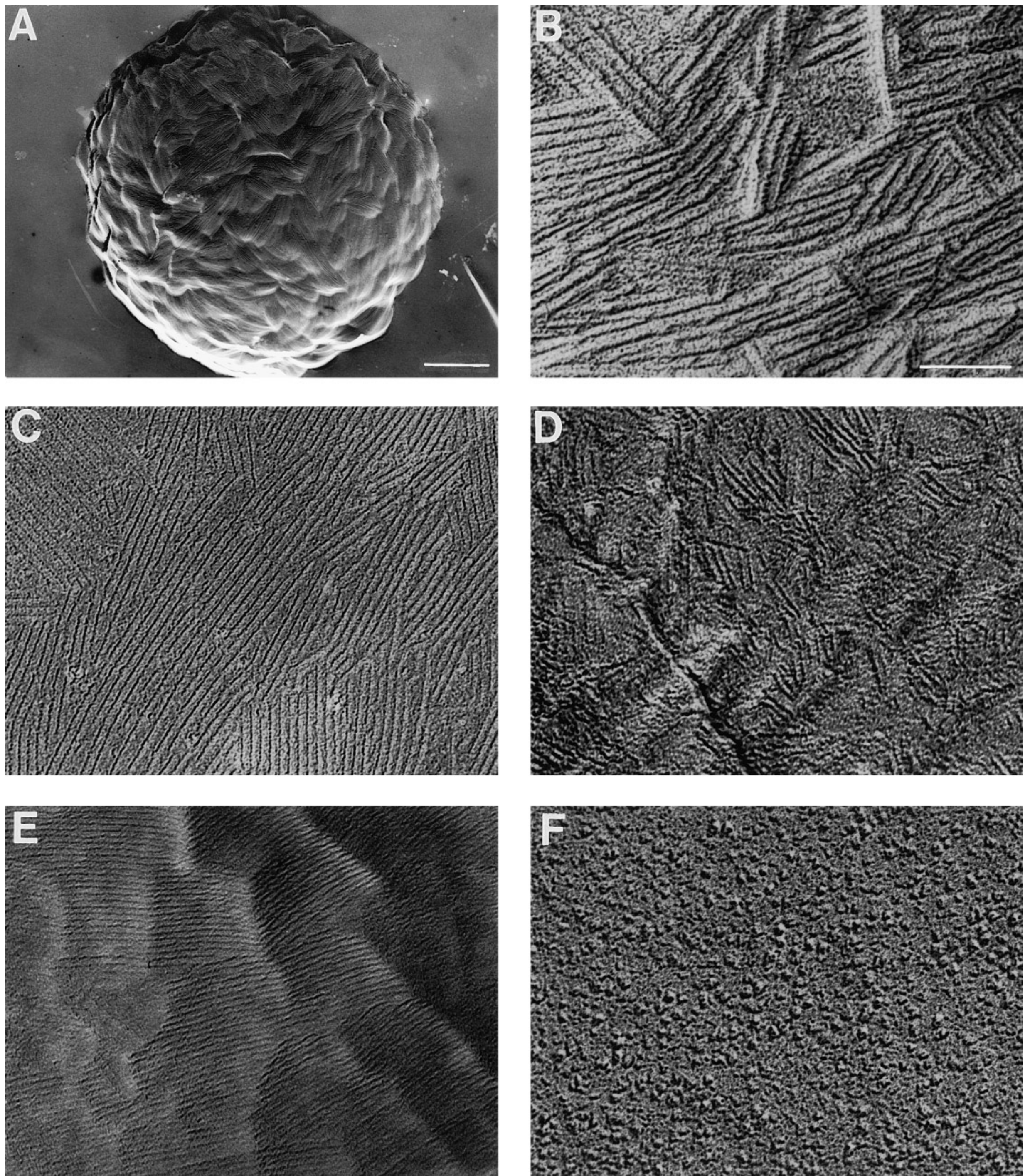

FIG. 3. Ultrastructural analysis of hydrophobin-encoded rodlet layers. Transmission electron micrographs of carbon–platinum replicas of the surfaces of fungal structures. B–F are all at the same magnification to allow comparison. (A) Tip of a conidium of *Penicillium notatum,* freeze-fractured. Bar, 500 nm. (B) Surface of conidium of *Neurospora crassa* showing the *EAS*-encoded rodlet layer, freeze-fractured. Bar, 100 nm. (C) Surface of conidium of *Aspergillus nidulans* showing the *rodA*-encoded rodlet layer, freeze-fractured. (D) Surface of the pileus of *Agaricus excellens* mushroom showing rodlet layer. (E) Surface of conidium of *M. grisea.* Direct shadowing at room temperature to show fine structure of surface rodlets encoded by *MPG1.* Note the bootlace internal structure on rodlets. (F) Surface of a conidium of an *mpg1*$^{-}$ null mutant showing the absence of a rodlet layer.

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hyphal surfaces often exceed 120°. Easily wettable phenotypes associated with hydrophobin mutants show that rodlet layers are instrumental in generating surface hydrophobicity, but the significance of the rodlet patterning is less obvious. The individual rodlets are too small to contribute to hydrophobicity because topological features need to exceed 100 nm in order to do this, and rodlets are seldom more than 15 nm in diameter (Wessels, 1997). Nevertheless, whether hydrophobicity is altered by the presence of rodlets on fungal surfaces, particularly in interactions with microdrops of water or heterogeneous surfaces, is uncertain and hard to prove experimentally. Spore rodlets have also been assumed to serve a role in dispersal and protection of spore contents from desiccation (Beever and Dempsey, 1978) but this has not been confirmed experimentally.

Whether surface rodlet layers are exclusively composed of hydrophobin aggregates is also uncertain. Rodlets identified by drying solutions of purified Sc3 hydrophobin resemble hyphal rodlet layers from *S. commune,* but are qualitatively different, particularly in terms of the length of individual rodlets, although length was found to be altered by the concentration of hydrophobin monomers (Wösten *et al.,* 1994a). Furthermore, the presence of more than one hydrophobin in the spore walls of *A. nidulans* suggests that rodlet layers might be composed of aggregates of more than one protein, although the absence of a rodlet layer in *A. nidulans rodA*$^-$ mutants, still expressing *dewA,* suggests that the *rodA*-encoded hydrophobin is the primary rodlet component. The presence of three potential myristylation motifs in the *EAS* hydrophobin and the homology to plant lipid-binding proteins also suggests that lipid interactions are involved in rodlet layer production or their linkage to the underlying cell wall (Bell-Pederson *et al.,* 1992; Lora *et al.,* 1995). Hydrophobins can be glycosylated, and both Sc3 and a related hydrophobin from *Pleurotus ostreatus* have been shown to be conjugated with carbohydrate (S. A. Asgeirsdottir and J. G. H. Wessels, personal communication). The cryparin hydrophobin has also been shown to have lectin-binding activity (Carpenter *et al.,* 1992), indicating that carbohydrate interactions may also be involved in building the characteristic hydrophobin layers.

The Diversity of Fungal Hydrophobins

Rodlet proteins have been recognized in fungi for many years and show considerable diversity among different species. This is evident among the *Penicillia,* where Hess *et al.* (1968) showed, in an elegant series of studies, that rodlets could vary significantly in diameter, length, and overall architecture. Figure 3 shows the diversity of hydrophobin-encoded rodlets layers from conidia of *Penicillium notatum, A. nidulans, N. crassa,* and *Magnaporthe grisea* and the pileus of the mushroom *Agaricus excellens.* Rodlet layers are common features of a number of other microorganisms, including aerial structures of filamentous actinomycetes. In *Streptomyces coelicolor,* for example, rodlets are seen on spores and aerial hyphae and strongly resemble those of filamentous fungi (Williams *et al.,* 1972).

In addition to forming surface rodlet layers, hydrophobins form layers designed to seal hyphal aggregations in more complex structures. This appears to be one of the roles of Sc4 in the basidiomes of *S. commune.* Here the hydrophobin lines internal air spaces and gaps between aggregated hyphae (Wessels, 1997). Rodlet layers may therefore link fungal hyphae to one another as well as to other substrates. This is significant because complex fungal structures require hyphal aggregation and the provision of gas channels within tissue. In this context rodlet layers have also been seen lining the gas channels found in lichen thalli (Honegger, 1993).

The role of a number of other abundantly expressed Class I and II hydrophobins recently identified from both ascomycete and basidiomycete fungi (Table 1) remains unclear as mutant phenotypes have yet to be reported. Expression patterns and immunolocalization experiments suggest that many are involved in forming surface layers on aerial structures. These include the product of *ABH1* from the cultivated mushroom *Agaricus bisporus,* which is present on the surface of mushroom caps and lining internal gas channels within the mushroom body (Lugones *et al.,* 1996). A selection of hydrophobins are abundantly produced during vegetative growth of fungi, including *HFB1* from *Trichoderma reesei* (Nakari-Setälä *et al.,* 1996). These hydrophobins may be required for attachment to solid substrates or for aerial development, but await further characterization.

THE ROLE OF HYDROPHOBINS IN FUNGAL PATHOGENICITY

The potential importance of hydrophobins to pathogenic fungi has been shown in a number of independent studies. Cerato-ulmin was extensively studied as a phytotoxin before it was found to be a hydrophobin (Takai, 1974, 1980). The protein was shown to have phytotoxicity (Okamoto *et al.,* 1986), but its production could not be consistently associated with aggressive pathogenicity of

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isolates (Brasier *et al.,* 1995). Gene disruption of the cerato-ulmin-encoding gene showed that the protein cannot be considered a pathogenicity determinant because deletion mutants were still able to cause disease (Bowden *et al.,* 1996). The experiments did, however, highlight the relatedness with other hydrophobins because cerato-ulmin mutants were reduced in aerial hyphae formation and surface hydrophobicity. Consistent with this, cerato-ulmin is a component of the cell wall of *O. ulmi* (Takai and Hiratsuka, 1980, 1984) and is a surface-active protein (Russo *et al.,* 1982; Stickler and Bolyard, 1994). Very recent information has suggested that cerato-ulmin may be involved in spore adhesion to the bark beetles which transmit *O. ulmi* (Temple *et al.,* 1997). This may explain both the abundant secretion of cerato-ulmin during pathogenesis and its correspondence with pathogenicity.

A protein similar to cerato-ulmin has been identified in the horse chestnut blight fungus *Cryphonectria parasitica.* The hydrophobin, called cryparin, is produced abundantly during growth of the pathogen (Carpenter *et al.,* 1992). Down-regulation of the cryparin-encoding gene *CRYP* during virus-mediated hypovirulence has suggested a role for cryparin in pathogenesis (Zhang *et al.,* 1994; Kazmierczak *et al.,* 1996), but this has not been fully established and it appears likely that the role of cryparin is similar to that of cerato-ulmin in either substrate attachment or aerial morphogenesis.

A study clearly implicating hydrophobins in fungal pathogenesis was an investigation that identified genes of the rice blast fungus *Magnaporthe grisea* expressed during its colonization of rice tissue. This led to isolation of *MPG1,* a gene highly expressed during two stages of pathogenesis: during appressorium elaboration and during symptom development (Talbot *et al.,* 1993). Gene replacement of *MPG1* resulted in a reduction in the number of disease lesions produced by the fungus on rice leaves, due to a reduction in appressorium formation on the rice leaf surface (Talbot *et al.,* 1996). *MPG1* encodes a typical Class I hydrophobin which appears to self-assemble in response to hydrophobic surfaces (Talbot *et al.,* 1996). MPG1p is therefore likely to have a function in the perception of inductive surfaces for the initiation of appressorium development (Talbot, 1995; Beckerman and Ebbole 1996; Talbot *et al.,* 1996). This is likely to be a consequence of the ability of MPG1p to self-assemble at a hydrophobic surface (Talbot *et al.,* 1996), but it is unclear whether this hydrophobin is especially adapted for self-assembly at rice leaf surfaces or merely expressed at an appropriate time. The function of *MPG1* in appressorium development suggests that hydrophobins function in the intimate association between fungi and the substrates upon which they subsist (Wösten *et al.,* 1995; Beckerman and Ebbole, 1996; Talbot *et al.,* 1996). The role of *MPG1* also shows how surface associations can have important developmental consequences: in this case the correct conformation of an outer cell wall layer is required for efficient initiation of cellular differentiation.

The discovery of *MPG1* and cerato-ulmin has led to the identification of many other hydrophobins from pathogenic fungi and in at least one case—the tomato pathogen *Cladosporium fulvum*—there appears to be an association between pathogenesis and production of hydrophobins (Spanu, 1997). In other studies, including those of fungal pathogens of animals, the association has not been seen (Parta *et al.,* 1994; Thau *et al.,* 1994). Hydrophobins may therefore have been coopted by certain pathogenic fungi for surface interactions associated with fungal infection, but cannot at this stage be considered general pathogenicity determinants because they clearly display a range of functions in different fungi. An interesting example illustrating hydrophobin diversity is the discovery of an unusual hydrophobin from *Claviceps purpurea,* the causal agent of Ergot disease of rye (Arntz and Tudzynski, 1997). A hydrophobin-encoding gene, *cpa3,* was found in a cDNA screen to identify genes expressed during alkaloid production by the fungus. The predicted amino acid sequence of *cpa3* contained three repeated hydrophobin (eight-cysteine) domains within a single open reading frame, separated by glycine–asparagine repeats. Each of these Class II hydrophobin domains strongly resembled one another and showed homology to cerato-ulmin and cryparin (the latter hydrophobin also possesses a similar glycine rich N-terminus, as shown in Fig. 1). Because alkaloid formation occurs at the same time as sclerotial formation, this implicates the product of *cpa3* in formation of these structures, perhaps by cementing hyphae together. The discovery of *cpa3* emphasizes the diverse morphogenetic processes likely to be mediated by hydrophobins, many of which may be fundamental to the success of pathogenic species.

REGULATION OF HYDROPHOBIN GENE EXPRESSION

As illustrated above, hydrophobins are involved in a variety of morphogenetic processes and, as such, their production is subject to complex regulation. The investiga-

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tion of hydrophobins as morphogenetic target genes has often illuminated underlying regulatory pathways involved in fungal cell differentiation (Stringer *et al.,* 1991; Bell-Pederson *et al.,* 1996). An example is the *rodA* gene, which is regulated by the central regulator of *brlA* (Stringer *et al.,* 1991) but independently of "downstream regulators" such as *wetA* and *abaA.* In contrast, *dewA* is regulated by the product of *wetA,* which is consistent with its expression after the formation of conidia (Stringer and Timberlake, 1995). Use of the *rodA* gene as a morphogenetic target led to the identification of *cis*-acting elements responsive to *brlA* known as bristle response elements (BREs), which have since been recognized in a number of *A. nidulans* genes regulated by *brlA* (Chang and Timberlake, 1993).

A promoter analysis of *EAS/ccg-2* revealed *cis*-acting elements which regulate expression of the gene in response to circadian patterns, light, and nutrient starvation (Bell-Pederson *et al.,* 1996). The *EAS* gene is expressed during the morning which is controlled by a region of DNA known as the activating clock element. This gene is subject to repression during mycelial growth and positive control by blue light and by a transcriptional activator involved in conidiation. A mutation at the corresponding *cis*-acting site is in fact the cause of the original *eas* mutant (Selitrenikoff, 1976; Beever and Dempsey, 1978; Kaldenhoff and Russo, 1993). The *Sc1, Sc4,* and *Sc6* genes are regulated by mating-type genes being limited to the dikaryotic phase of growth. Expression of *Sc1, Sc4,* and *Sc6* also requires the action of the *FBF* gene for dikaryon-specific expression, although this does not induce Sc3 expression, consistent with high-level expression in monokaryons (Wessels, 1997). All the *S. commune* hydrophobins are regulated by the *THN*-encoded regulator, which controls aerial development and emergent growth in the fungus (Wessels *et al.,* 1991b).

MPG1 is regulated by starvation stress and induced by either carbon source or nitrogen source starvation (Talbot *et al.,* 1993). *MPG1* is regulated by the wide domain regulator of nitrogen starvation *NUT1* (homologous to *areA* and *nit2* of *A. nidulans* and *N. crassa,* respectively). It is also regulated by two novel *trans*-acting regulators encoded by the *NPR1* and *NPR2* genes (Lau and Hamer, 1996). *NPR1* and *NPR2* regulate the response to nitrogen starvation and/or carbon starvation but are also required for pathogenicity of *M. grisea.* It is therefore likely that they encode regulators of pathogenic specialization by *M. grisea* and its ability to grow in living plant tissues. An analysis of the *MPG1* promoter revealed the presence of four GATA-like binding sites which may be recognized by *NUT1* (Lau and Hamer, 1996; Talbot, unpublished). Interestingly a number of near-consensus BREs were also found, implying that *MPG1* may be regulated by a *brlA*-homologous regulator during conidiation and perhaps appressorium development (Talbot, unpublished).

The complexity of regulation, where single hydrophobin genes are regulated by multiple *trans*-acting factors, mirrors the number of environmental contingencies monitored by fungi for developmental processes, such as conidiation, to proceed. It also emphasizes the flexibility of control of fungal morphogenesis. Hydrophobin genes have been extremely useful tools for studying morphogenesis because of their regulation during cellular differentiation, and identification of regulatory pathways controlling fungal development may be a use for which hydrophobin genes are ideally suited.

REPELLENT PROTEINS

The recent identification of a repellent protein in *Ustilago maydis* has illustrated that fungi have evolved other mechanisms for making hyphae hydrophobic in addition to hydrophobins. The identification of repellent in *U. maydis* resulted from an investigation into the role of hydrophobins in this dimorphic plant pathogen. *U. maydis* is an important pathogen causing smut disease of maize and has also emerged as a tractable model for understanding the biology of self/nonself recognition in fungi (Banuett, 1995). The fungus can live either as a nonpathogenic yeast or in a pathogenic filamentous form. Because of the role of filamentous growth in conditioning pathogenicity and since this is the only stage which undergoes aerial morphogenesis, it is ideally suited for investigating hydrophobin function. However, the recent study by Wösten and colleagues (1996) revealed a more interesting feature, namely, the discovery of a completely new class of proteins which may perform a similar function to hydrophobins.

To grow as a filamentous mycelium, *U. maydis* requires heterozygosity at two mating type loci, *a* and *b.* The *a* locus has two alleles, *a1* and *a2,* which control fusion of sporidia by production and detection of pheromones, while the multiallelic *b* locus controls all postfusion events. To isolate hydrophobin-like proteins Wösten *et al.* carried out extractions from a filamentous diploid strain (*a1 a2 b1 b2*) and a near-isogenic homozygous diploid (*a1 a2 b2 b2*) which can grow only as a yeast. Cell walls from both strains were extracted with hot detergent and TFA, and proteins examined after fractionation through denaturing gels. A

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highly abundant 8-kDa protein was found in the detergent-insoluble TFA-extractable fraction, which was insoluble in water or aqueous ethanol and specific to the filamentous diploid strain. The purified protein, named rep1-2, was partially sequenced and the *rep1* gene cloned and characterized. The gene structure of *rep1* showed the presence of a typical secretion signal followed by 12 repeated sequences of between 37 and 55 amino acids within a large open reading frame of 652 residues. The primary amino acid sequence and organization of rep1 are therefore completely unrelated to those of fungal hydrophobins. The rep-1 repeated sequences have a consensus of 37 conserved amino acids of alternating hydrophobic and hydrophilic pattern and the whole gene product possesses 32.9% hydrophobic residues regularly spaced within these repeats. The presence of the much smaller 8-kDa rep1-2 protein suggested considerable posttranslational modification of the *rep1* gene product, which was confirmed by the discovery of 10 KEX2-like proteolytic cleavage sites (Bostian *et al.,* 1984; Fuller *et al.,* 1988) within the sequence. The *rep1* gene therefore putatively encodes rep1-2 and nine additional processed proteins ranging in size from 35 to 53 amino acids. Eight of these peptides were detected by HPLC fractionation of the cell wall TFA extracts, confirming that complex posttranslational modification is involved in repellent function.

Disruption of the *rep1* gene produced mutants which could still form filamentous dikaryons, but hyphae were not able to grow into the air or to spread from an aqueous environment along a dry hydrophobic surface. The mutant phenotypes are therefore not dissimilar to those found in the *sc3*$^-$ mutants of *S. commune* and it may be worth speculating that repellents in *U. maydis* are able to fulfill a biological function very similar to that of hydrophobins found in other fungi (Wösten *et al.,* 1996). The amphipathic nature of rep1 oligopeptides, and the mutant phenotypes observed, indicates that repellents may even act in a manner similar to hydrophobins. It appears likely, for example, that they form a component of the walls of aerial hyphae, but rather than self-assembling when exposed to an interface it is possible that repellent oligopeptides aggregate, aligning with their hydrophobic sides in contact with the external environment and hydrophilic sides facing the underlying cell wall (for model see Talbot, 1997). This is, at present, a speculation and further work is needed to determine the mechanism by which repellents function. It is also possible, for example, that hydrophobins and repellents interact within fungal cell walls to condition hydrophobicity, with the repellents perhaps anchoring hydrophobins in position. A hydrophobin is also known in *U. maydis* (Bohlmann, 1996), but its role in aerial hyphae formation has not been established. The ease of manipulation of *U. maydis* coupled with the dimorphic life cycle of the fungus make it ideal for studying the interplay between hydrophobins and repellents and their respective roles in aerial morphogenesis.

The discovery of repellents also suggests a possible link between aerial morphogenesis in eukaryotic and prokaryotic microorganisms. This is because the structure of processed repellent oligopeptides is reminiscent of that of SapB, a small morphogenetic protein identified in aerial hyphae of the filamentous bacterium *Streptomyces coelicolor* (Willey *et al.,* 1991). SapB is an 18-amino-acid protein containing 38.9% hydrophobic residues and may be synthesized by enzymatic (nonribosomal) protein synthesis. Purified SapB peptide can cross-complement *bld* mutants blocked in the production of hydrophobic aerial hyphae (Willey *et al.,* 1991, 1993). Because of these similarities it may be worth speculating that both SapB- and repellent-mediated aerial development involve anchoring of an amphipathic protein layer, built of many oligopeptide subunits, to aerial hyphae. Interestingly, it has been shown that *S. coelicolor bld* mutants can be complemented with purified Sc3 hydrophobin (R. D. Tillotson, H. A. B. Wösten, and J. M. Willey, personal communication), showing that the physical requirements for building aerial hyphae—namely, lowering surface tension—are the same in filamentous prokaryotes and eukaryotes even though the mechanisms by which this is achieved may be distinct.

APPLICATIONS

The unusual features of hydrophobins and repellents suggest that they will have a number of industrial applications. Potential applications of hydrophobins are described in some detail by Wessels (1996, 1997) and are based largely on the surface activity and self-polymerization properties described earlier. Repellents will require greater characterization before applications can be predicted, but the amphipathic natures of these oligopeptides suggest that they too may be useful in altering the properties of surfaces to which they are applied.

Because hydrophobins self-assemble in response to aeration they have been shown to coat gas vesicles, altering their shape and stability (Wösten *et al.,* 1993). These form very stable foams under laboratory conditions (Wösten *et al.,* 1993; Talbot, unpublished observations). The use of

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hydrophobins may therefore provide a natural improvement to the ability of foods to react to phase changes and to form stable foams. This characteristic is important in a variety of food manufacturing processes, such as the production of cream products, confectionery, soft drinks, and beer. Furthermore, Class II hydrophobins, which also produce stable foams on aeration, show a solubility difference when exposed to pressure (Takai and Richards, 1978). This useful characteristic suggests that they may be effective in pressurized food products where formation of a stable foam is required upon release from a pressurized container.

Hydrophobins also alter the wettability of surfaces (Wösten *et al.,* 1993; Talbot *et al.,* 1996), which may allow their use in the manufacture of various products. For example, natural surface-active agents are useful in hair care products and as primers for adhesives. Self-assembly on plastic surfaces can also be used to change surface properties, such as water migration, or for the provision of molecular anchor points for other biochemical functions. A natural extension of these applications is the use of hydrophobins in the medical industry for coating plastic surfaces with a natural protein layer which may be useful for transplantation surgery or implanting. The surface activity and spontaneous self-assembly of hydrophobins therefore make them potentially very useful proteins for many processes.

Another significant characteristic of hydrophobins in any of these processes is that humans have been unwittingly ingesting them for many years (Wessels, 1996), because hydrophobins coat the surfaces of cultivated mushrooms (de Groot *et al.,* 1996; Lugones *et al.,* 1996). This suggests that they are nontoxic natural products that will be safe to introduce into a variety of products.

FUTURE PERSPECTIVES

The fungal hydrophobins and repellents deserve future study for a number of reasons. First and foremost, they will supply fundamental information into the mechanism by which fungi generate upwardly projecting aerial hyphae. Because this ability underpins much of fungal development it will be of great significance in understanding the biology of fungi. In this context a number of areas will require attention. How, for example, are hydrophobins attached to the underlying cell wall and how is their secretion and subsequent self-assembly temporally regulated with cell wall biosynthesis? It will also be informative to define the function of spore rodlet layers in more detail and to determine how these layers are laid down during conidiogenesis, how their production is regulated with the onset of dormancy, and how such strong protein coats are disrupted during spore germination?

The evolution of hydrophobins and repellents is also a fascinating avenue for future research. It appears, for example, that hydrophobins are ubiquitous and yet extremely diverse. The limited set of genetic studies, however, indicates some conservation of basic function: most hydrophobins appear to condition the hydrophobic character of external fungal surfaces. The different functions may therefore reflect the developmental stage at which they are produced. If this is true, then one could predict that any hydrophobin would be able to complement the mutant phenotype of any other—as long as it is expressed at the appropriate developmental stage. Conversely, the considerable divergence in hydrophobin sequence could reflect real functional diversity. If this is the case, then complementation of hydrophobin mutant phenotypes would perhaps be restricted to those proteins occupying the same role in different fungi. Investigating hydrophobin function by using this cross-species complementation approach may therefore be the most informative way of determining the evolutionary and functional relatedness of hydrophobins. Preliminary results from such an investigation in our laboratory point to the latter situation, with hydrophobins occupying quite specific roles (Kershaw and Talbot, unpublished).

The importance of aerial development to fungi is highlighted by the observation that structurally distinct proteins have been recruited to make the surface of hyphae hydrophobic. This is not surprising given the overriding importance of aerial morphogenesis to spore production and dissemination by terrestrial fungi. The identification of a number of pathways for aerial development in filamentous bacteria, (Willey *et al.,* 1993) and the greater complexity of fungi, suggests that more processes will emerge. It may be that study of hydrophobins and repellents from the same species will therefore be instrumental in determining the regulation of aerial development and perhaps the evolutionary relationship between the two types of proteins. The pioneering study of *U. maydis* will doubtless be repeated in many other fungi shortly and will determine how widespread both classes of protein are and to what extent they have redundant functions or are cooperatively regulated. These studies should also determine whether there are similar underly-

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ing mechanisms to aerial morphogenesis in fungi and filamentous bacteria (see Talbot, 1997).

By far the most important avenue for future research will be the determination of a structure for both hydrophobins and repellents. So far, the structure of a hydrophobin has been difficult to elucidate due to their great propensity for aggregation, but significant effort must now be applied to determine a structure either crystallographically or by NMR. This will need to be coupled with mutagenic studies and complementation of the interesting and diverse hydrophobin mutant phenotypes if a full understanding of the biology of these proteins is to emerge. In the short term, a comparative analysis with plant disulfide-rich proteins (Baud *et al.,* 1993) may prove a useful guide to future site-directed mutagenesis studies.

The application of hydrophobins to industrial processes will also depend on structural information, but will also require development of a new method for hydrophobin purification which does not use agents such as TFA. This will be vital if industrial-scale production of hydrophobins is ever to be a reality and their unusual features exploited to the full.

In summary, hydrophobins are remarkable proteins which may prove to be key lifestyle determinants of fungi. Their discovery has already led to a significant advance in understanding fungal development and their future study offers great rewards.

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