

Fungal biology: Growing into the air

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Fungi must grow into the air for reproduction and spore dispersal, and to do this their hyphae contain morphogenetic proteins that respond to the aerial environment. The recent discovery of 'repellent' proteins, however, suggests fungi have more than one mechanism for aerial development.

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For microorganisms to colonize terrestrial habitats they must evolve effective mechanisms to prevent water loss from their cells. This is imperative, not only for them to subsist in the same environment during changeable prevailing conditions, but also for their offspring to spread to new habitats. Considering the overriding importance of this evolutionary adaptation to living on land, we know remarkably little about the molecular mechanisms by which terrestrial microorganisms grow into the air.

Fungi are prime examples of microorganisms that cope with diverse terrestrial ecosystems. Fungi grow by forming tubular, thread-like cells called hyphae that penetrate the substrates upon which they feed. In this way they are able to invade complex substrates very effectively, dissolving them with extracellular enzymes and transporting the resulting simple sugars or amino acids. It is this ability that has made them the principal degraders of biomass in the environment and also highly efficient pathogens of both animals and plants. They are perhaps best known to the casual observer, however, for their ability to form fuzzy aerial moulds on food sources and complex structures like mushrooms.

To form such aerial structures, fungi are able to break surface tension and grow into the air, an ability that is crucial to their survival on land. Once aerial structures are established, they are able to prevent excess water loss and can carry out their roles in sexual or asexual spore dispersal. Over the last three years, a body of information has emerged implicating a class of proteins called hydrophobins in these processes [1]. These proteins, which are able to react to interfaces between water and air, provide a hydrophobic covering for aerial fungal structures.

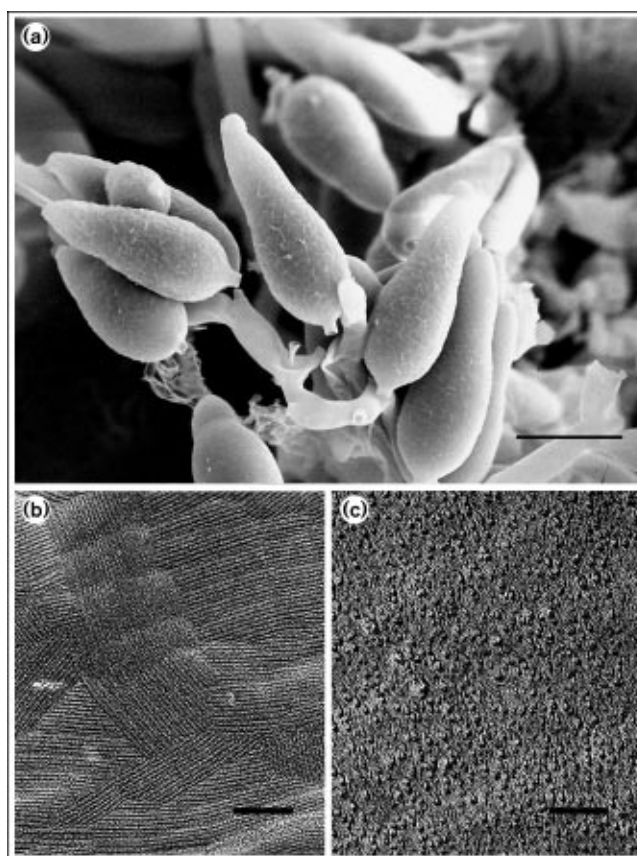
Fungal hydrophobins – proteins that react to interfaces

The fungal hydrophobins are small, secreted, moderately hydrophobic proteins with eight cysteine residues spaced

in a characteristic pattern in their amino-acid sequence. More than twenty hydrophobins have now been recognized and they may well prove to be ubiquitous in fungi [1]. In most cases, they have been identified as mRNAs abundantly transcribed during particular developmental processes such as sporulation, fruit-body formation or fungal infection of plants and insects. Genetic studies have now implicated hydrophobins in all of these morphogenetic processes.

Disruption of the *rodA* gene from *Aspergillus nidulans*, for example, or of the *EAS* gene from *Neurospora crassa*, produced mutants with spores which were 'easily-wetted', lacking the hydrophobic rodlet protein that normally protects them from desiccation and aids their dispersal [2,3].

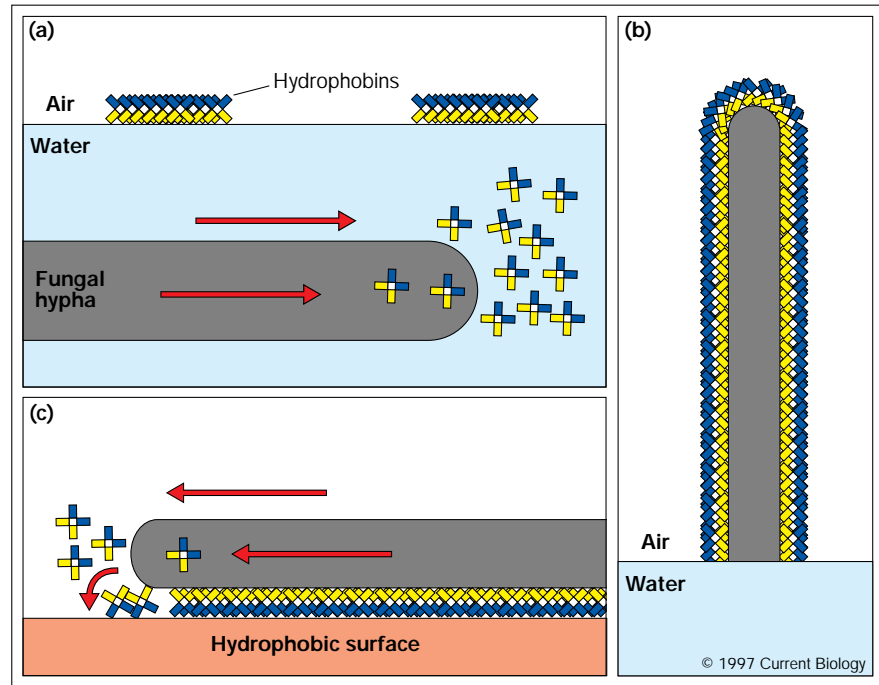
Figure 1



Hydrophobic rodlet layers are commonly found on aerial structures of fungi. (a) Conidiospores of the rice blast fungus *Magnaporthe grisea* (bar = 10 μm). (b) Rodlet proteins observed on the surface of these spores after freeze-fracture (bar = 100 nm). (c) The same surface in an *Mpg1* (hydrophobin-less) mutant (bar = 100 nm).

Figure 2

Model of the action of morphogenetic proteins during aerial development of fungi. Hydrophobins putatively contain four disulphide bridges, resulting in proteins with four external loops (represented as the four arms of a cross). The proteins fold so that one side is predominantly hydrophobic (blue) and the other hydrophilic (yellow); they are secreted when the fungus is in submerged conditions (a), but self-assemble when they reach air–water interfaces (b) or hydrophobic surfaces (c).



The hydrophobic rodlet layers produced by hydrophobins are common features of aerial structures of fungi, including mushroom caps, spores' surfaces and aerial hyphae themselves (Fig. 1). These layers seem to be directly composed of hydrophobins, because a solution of purified hydrophobin will dry into an amphipathic monolayer having the characteristic rodlet architecture on its hydrophobic side [4].

The process of self-assembly that leads to the formation of these rodlet polymers appears to involve the aggregation of hydrophobin monomers at air–water interfaces (Fig. 2). This process involves short-range hydrophobic interactions between the monomers, and the aggregates formed are extremely insoluble and not dispersed even in hot detergent extractions [4,5]. This property explains why hydrophobins, although often abundant, escaped detection for so long. Only by treating these insoluble aggregates with trifluoroacetic acid or formic acid can the polymers be dissociated into the component hydrophobin monomers [5]. It is this method that has enabled hydrophobins to be detected in diverse fungal species [1].

In the case of the mushroom fungus *Schizophyllum commune*, it was found that the hydrophobin Sc3 is secreted abundantly as a monomer in submerged liquid cultures, but self-assembles at air–water interfaces or after aeration of the culture [4]. The amphipathic polymers appear to act as foci for the formation of aerial hyphae, and continued secretion of the hydrophobin from the tips of

these hyphae ensures continual coverage of new growth with the hydrophobic layer. The hydrophilic side of the amphipathic hydrophobin layer would then become strongly connected with the underlying hyphal wall (Fig. 1b). The importance of this process in aerial development was shown by the way disruption of the *Sc3* gene generated mutants that lacked aerial hyphae, but could be complemented *in trans* using purified Sc3 protein [6].

The hydrophobin self-assembly process has other important consequences for fungal development. The *sc3*⁻ null mutants were found to be unable to adhere to hydrophobic surfaces, whereas purified Sc3 protein will self-assemble in response to surface interactions as well as aerial interfaces [7]. The importance of this ability is that it is often required for fungi to be able to undergo specific morphogenetic processes. In plant pathogenic fungi, for example, a crucial developmental response is their ability to make specialized infection cells that break plant cuticles using mechanical pressure or enzymatic action. The formation of these cells is often induced by very hydrophobic surfaces. In this context hydrophobins appear to act as sensors of hydrophobic surfaces, such as plant leaves, which are conducive to fungal infection [8].

The importance of hydrophobins in fungal development is therefore twofold. In order to produce fruiting bodies and sporulate effectively, most fungi need to grow into the air, while to feed effectively and invade substrates they must adhere closely to solid surfaces. Both processes

appear to involve hydrophobins. However, a number of conundrums exist. If hydrophobins are essential for aerial morphogenesis, then how do *sc3⁻* mutants still make aerial hyphae under certain circumstances [6]? Similarly, other hydrophobin-less mutants do appear to elaborate aerial hyphae, even though further hydrophobins have not been detected in these species [8]. It may be that other hydrophobins with subtly distinct properties await characterization in these species, but the overriding importance to fungi of surface and aerial growth suggests that other hydrophobin-independent processes must occur.

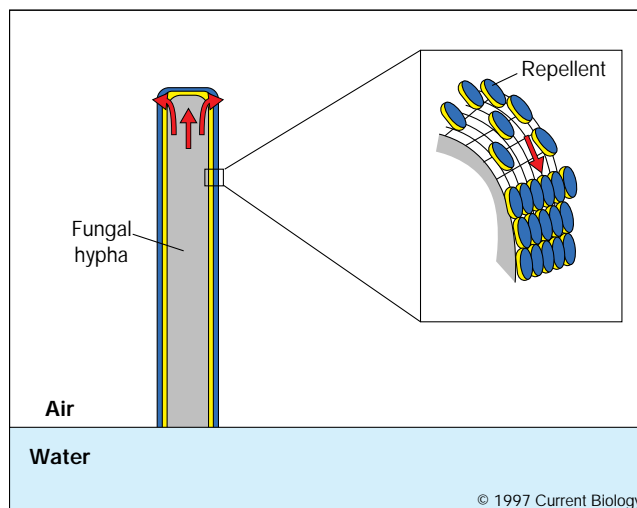
Repellents – small amphipathic morphogenetic proteins

The recent identification of novel proteins, the repellents, in *Ustilago maydis* [9] has shown that fungi have indeed evolved multiple mechanisms for aerial development. The identification of repellents in *U. maydis* was guided by both the unusual biochemical characteristics of hydrophobins and the dimorphic nature of this fungus, which makes it a perfect test organism for studying aerial morphogenesis. *U. maydis* causes corn smut disease, a serious condition causing tumours and black mould on corn cobs [10]. Interestingly, the fungus can only cause disease when it grows in a filamentous form, which occurs following fusion of haploid yeast-like sporidia to form a dikaryon on the leaf surface. This grows as a filamentous mycelium invading the underlying plant tissues. The formation of the filamentous mycelium is governed by two mating-type genes, *a* and *b*, requiring different alleles of both genes to be present for successful development. The *a* locus has two alleles, *a1* and *a2*, which control fusion of sporidia by production of pheromones, while the multi-allelic *b* locus conditions all post-fusion events including filamentous growth and pathogenicity [10].

To identify proteins specifically involved in the morphogenesis of filamentous hyphae Wösten and co-workers [9] carried out extractions from a filamentous diploid strain (*a1b1;a2b2*) and a near isogenic homozygous diploid (*a1b2;a2b2*), which can only grow as a yeast. Cell walls from both strains were extracted with hot detergent and trifluoroacetic acid, and proteins examined after fractionation through denaturing gels. Instead of observing hydrophobins (as expected) in the detergent-insoluble trifluoroacetic-acid-extractable fraction, they found a highly abundant 8 kDa protein. The purified protein, named rep1-2, was insoluble in water or aqueous ethanol and specific to the filamentous diploid strain. Partial amino-acid sequencing led to the design of degenerate oligonucleotide primers, and the *rep1* gene was subsequently cloned and characterized.

The sequence of the *rep1* gene is intriguing, revealing the presence of a typical secretion signal followed by twelve repeated runs of between 37 and 55 amino acids within an open reading frame of 652 residues. These repeats have a

Figure 3



Repellents are small amphipathic oligopeptides which may aggregate in response to interfaces, aligning their hydrophobic sides (blue) towards the external environment.

consensus of 37 conserved amino acids in an alternating hydrophobic and hydrophilic pattern (the complete protein has 32.9 % hydrophobic residues regularly spaced within these repeats). The much smaller size of the rep1-2 protein suggested that the primary *rep1* gene product undergoes considerable post-translational modification, and this inference was supported by the discovery of ten Kex2-like proteolytic cleavage sites within the sequence. The *rep1* gene putatively encodes rep1-2 and nine additional processed proteins, ranging in size from 35 to 53 amino acids. Eight of these peptides have been detected by the chromatographic fractionation of cell-wall trifluoroacetic acid extracts [9].

Disruption of the *rep1* gene has serious morphogenetic consequences in *U. maydis*. Although dikaryotic hyphae are still able to form, Wösten *et al.* [9] showed that these were not able to spread out from an aqueous environment along dry hydrophobic surfaces or into the air. Reduced aerial hyphae formation was also noted in diploids homozygous for the $\Delta rep1$ deletion allele. The amphipathic nature of the rep1-2 oligopeptides, and the mutant phenotypes observed, indicates that repellents may act in a similar way to hydrophobins. It is likely that they form a component of the walls of aerial hyphae, but that rather than self-assembling when exposed to an interface, they aggregate aligning with their hydrophobic sides in contact with the external environment and their hydrophilic sides with the underlying cell wall.

A model of the putative action of repellents is shown in Figure 3. In this way, repellents resemble SapB, a small morphogenetic protein identified in aerial hyphae of the

filamentous bacterium *Streptomyces coelicolor* [11]. SapB encodes an 18 residue protein containing 38.9% hydrophobic residues. Purified SapB can cross-complement mutants blocked in aerial development [11,12], and it may be that SapB-mediated and repellent-mediated aerial development both involve anchoring of an amphipathic protein layer, built of many oligopeptide subunits, to aerial hyphae.

Perspectives

It is clear from these recent studies that filamentous fungi have more than one type of morphogenetic protein that is capable of responding to external environmental conditions and generating surface hydrophobicity. This is apparent because, not only do *sc3*⁻ hydrophobin null mutants form aerial hyphae under certain environmental conditions, but *rep1*⁻ mutants also form sparse aerial hyphae at colony margins. It is not surprising that such a fundamental ability of fungi has more than one underlying mechanism, and it seems likely that differing environmental factors may induce the activity of specific morphogenetic proteins. This is certainly paralleled in *S. coelicolor*, where SapB-independent mechanisms for aerial development also exist [12].

The exciting and immediate prospect is the characterization of functional redundancy between hydrophobins and repellents. Does *U. maydis*, for example, contain hydrophobins, and if so is their role distinct from that of the repellent *rep1-2*? Can purified hydrophobins cross-complement repellent-deficient mutants, and *vice versa*, to allow aerial morphogenesis? And are there general principles underlying the capacity to form upwardly projecting hyphae in both fungi and filamentous prokaryotes? It is very likely that the procedures pioneered by Wösten *et al.* [9] will be widely repeated in the near future. The deeper challenge, however, will be to explore the dynamics of self-assembly and aggregation to determine the molecular basis of interface perception by fungi.

Acknowledgements

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