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REVIEW ARTICLE

Surface-active proteins enable microbial aerial hyphae to grow into the air

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Overview

Although filamentous bacteria (i.e. the streptomycetes) and filamentous fungi belong to different kingdoms that diverged early in evolution, they adopted similar life styles. Both groups form aerial structures from which (a)sexual spores can develop. Some of the key processes involved in the formation of aerial hyphae by these microbes appear to be very similar. Both groups secrete highly surface-active molecules that lower the surface tension of their aqueous environment enabling hyphae to grow into the air. In the case of filamentous bacteria, small peptides (i.e. SapB and streptofactin) are secreted, while filamentous fungi use proteins known as hydrophobins to decrease the water surface tension. Although these fungal and bacterial molecules are not structurally related, they can, at least partially, functionally substitute for each other. Once escaped into the air, hyphae are covered with a hydrophobic film. In both filamentous fungi and filamentous bacteria this film is characterized by a mosaic of parallel rodlets. In fungi, this is the result of the self-assembly of hydrophobins but their bacterial equivalents have not yet been identified.

Background

Filamentous fungi and filamentous bacteria (i.e. actinomycetes, which include *Streptomyces* spp.) display a very similar lifestyle. In fact, it was only in the 1950s that it was established that these groups belong to different kingdoms (Goodfellow *et al*., 1983). Both filamentous fungi and filamentous bacteria occur in a wide variety of natural and man-made habitats. They are saprotrophic, and can be pathogens or symbionts of plants or animals. Representatives of both groups initially form a branched submerged mycelium consisting of filaments that are surrounded by rigid cell walls. These filaments grow at their apices and secrete large amounts of enzymes that degrade polymeric substrates (e.g. plant material) into small molecules that serve as nutrients. After a substrate feeding mycelium has been established, both filamentous fungi and filamentous bacteria may leave the hydrophilic environment to form aerial hyphae that may further differentiate to simple or elaborate spore-bearing aerial structures. Aerial hyphae of most species are hydrophobic and are characterized by rodlet-decorated surfaces. In this article we will describe observations that show that growth of aerial hyphae in fungi and bacteria involves surprisingly similar mechanisms. Although the molecules involved in aerial growth (i.e. SapB, streptofactin and hydrophobins) are not homologous, they can, at least partially, functionally substitute for each other. We have not extensively discussed the biochemical and biophysical properties of these surface-active molecules, although their characterization was instrumental in establishing their role in aerial growth. For this, we refer to recent reviews by Wösten *et al*. (1999a) and Wösten & de Vocht (2000).

Surface-active proteins involved in formation of aerial hyphae in filamentous fungi

Hydrophobins have been identified as proteins involved in fungal emergent growth. They are small (~100 aa) secreted proteins characterized by similar hydropathy patterns and by the presence of eight conserved cysteine residues (Wessels, 1997). Class I hydrophobins have the remarkable capacity to self-assemble at any hydrophilic–hydrophobic interface into a 10 nm thick amphipathic protein film. The hydrophobic side of such films typically features a mosaic of parallel rodlets (Wösten *et al*., 1993, 1994b, 1995). Self-assembly at the water–air interface is accompanied by a dramatic decrease in the water surface tension. For instance, self-assembled SC3 hydrophobin of the basidiomycete *Schizophyllum commune* reduces the water surface tension from 72 to as low as 24 mJ m^{-2}, making it the most surface-active protein identified to date (Wösten *et al*., 1999b).

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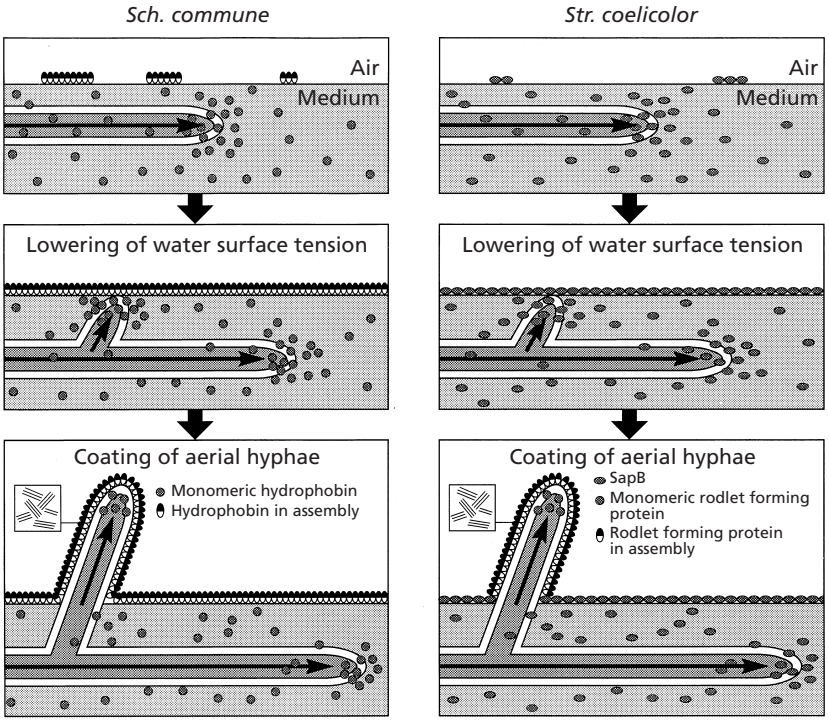

Fig. 1. Model for the formation of aerial hyphae in the filamentous fungus *Sch. commune* and the filamentous bacterium *Str. coelicolor*. After a submerged feeding mycelium has been formed, *Sch. commune* secretes SC3 into the medium, while *Str. coelicolor* produces SapB. These molecules lower the surface tension of the aqueous environment enabling hyphae to escape the substrate and to grow into the air. SC3 lowers the surface tension by assembling into an amphipathic membrane at the water–air interface. SC3 secreted by aerial hyphae of *Sch. commune* assembles at the interface between the hydrophilic cell wall and the hydrophobic air exposing its hydrophobic side, which is characterized by a mosaic of rodlets. The hydrophobic surface of aerial hyphae of *Str. coelicolor* is also typified by a rodlet layer. Although the molecules forming this layer have not yet been identified, evidence suggests it is not SapB.

Hydrophobins fulfil a broad spectrum of functions in fungal development. They mediate escape of fungal hyphae from their hydrophilic environment, coat fungal surfaces exposed to the air with a hydrophobic membrane and mediate attachment of hyphae to hydrophobic surfaces (Wessels, 1997; Wösten & Wessels, 1997; Talbot, 1997; Wösten *et al*., 1999b).

The role of hydrophobins in the formation of aerial hyphae is exemplified by the class I hydrophobin SC3 of *Sch. commune*. Like other filamentous fungi, *Sch. commune* colonizes moist substrates. After a feeding submerged mycelium has been established this fungus can form sterile aerial hyphae and fruiting bodies that are involved in sexual reproduction. In erecting these aerial structures, the fungus is confronted with the high surface tension of the water film surrounding the hyphae. To escape the barrier of the water surface tension (72 mJ m^{-2}), *Sch. commune* secretes the SC3 hydrophobin which lowers the water surface tension enabling hyphae to breach the interface and to grow into the air (Fig. 1; Wösten *et al*., 1999b). Young cultures not yet forming aerial structures do not express the *SC3* gene and the water surface tension remains high. After 3 d growth, the first SC3 monomers are secreted into the culture medium and the surface tension drops to 45 mJ m^{-2}, correlating with the emergence of the first aerial hyphae. Most aerial hyphae are formed after 4 d growth, which is accompanied by a high level of *SC3* gene expression and a maximal drop in the medium surface tension to 27 mJ m^{-2}. In an isogenic strain of *Sch. commune* in which the *SC3* gene is disrupted (strain ΔSC3) (Wösten *et al*., 1994b; van Wetter *et al*., 1996), surface tension decreases maximally to only 45 mJ m^{-2} and few aerial hyphae are observed (Wösten *et al*., 1999b). However, the decrease in surface tension and the formation of aerial hyphae are restored by the addition of purified hydrophobin to the culture medium. The observation that in both the wild-type and the ΔSC3 strain aerial hyphae begin to emerge at a surface tension of 45 mJ m^{-2} indicates that this is a critical surface tension that allows hyphae to breach the interface (Wösten *et al*., 1999b). At this surface tension, hyphae probably grow perpendicular to the interface, while hyphae that escape the interface at a lower surface tension may have met the surface at a lower contact angle.

Strain ΔSC3 does not form aerial hyphae in a liquid standing culture. However, when grown on solid medium the phenotype is less severe (van Wetter *et al*., 1996). This is probably due to a low level of the SC4 hydrophobin that is produced under these conditions. When this hydrophobin was expressed behind the *SC3* promoter, formation of aerial hyphae in the ΔSC3 strain was restored (van Wetter *et al*., 2000). In contrast, when expression of *SC4* was reduced by increasing the carbon dioxide concentration, formation of aerial hyphae was strongly reduced in the ΔSC3 strain.

Because the tips of aerial hyphae are isolated from the aqueous milieu of the feeding mycelium, SC3 secreted at the tips of such hyphae does not diffuse into the medium. Instead, SC3 monomers self-assemble at the interface between the hydrophilic cell wall and the hydrophobic air, forming an amphipathic film that coats the emergent aerial hyphae while at the same time conferring hydrophobicity to the hyphal surface (Fig. 1; Wösten *et al*., 1994a). Self-assembled SC3 imparts a characteristic mosaic of parallel rodlets found on the surface of

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emergent hyphae (Wösten *et al*., 1993). However, only a fraction of the total hydrophobin produced is secreted by the hyphal tips of the emergent filaments. The majority of SC3 is secreted by submerged hyphae. It remains in the medium where it only facilitates hyphal erection into the air. To be coated with a hydrophobic film, emergent hyphae must themselves secrete SC3 (Fig. 1; Wösten *et al*., 1999b).

If lowering the surface tension is required to allow hyphae to grow into the air, one would expect that other surface-active molecules would also be effective. Many surfactants, however, interfere with the plasma membrane and are therefore toxic (Lang & Wagner, 1993). Indeed, of all surfactants tested, only the fungal class I hydrophobins SC4 and ABH3, which are known to self-assemble, and the bacterial peptide streptofactin of *Streptomyces tendae* restore formation of aerial hyphae in the ΔSC3 strain (Wösten *et al*., 1999b; Lugones *et al*., 1998). The capacity of fungal hydrophobins to lower the water surface tension is the result of a conformational change that occurs when they self-assemble at the water–air interface (van der Vegt *et al*., 1996; de Vocht *et al*., 1998). The resulting amphipathic film is not expected to diffuse through the cell wall and to interfere with the plasma membrane, which could explain their nontoxicity and their capacity to facilitate hyphal emergence. We expect that the model as presented for *Sch. commune* is a general mechanism for fungal aerial growth. Other fungi also secrete hydrophobins into the medium (see Wösten & Wessels, 1997), one of which, the ABH3 hydrophobin of *Agaricus bisporus*, restores lowering of the surface tension in a ΔSC3 strain (Lugones *et al*., 1998). A similar mechanism is thought to account for the capacity of bacterial amphipathic peptides to facilitate aerial growth (Tillotson *et al*., 1998; Richter *et al*., 1998).

Are hydrophobins the only class of structural proteins involved in formation of fungal aerial hyphae? Other proteins in the medium of a monokaryon of *Sch. commune* did not complement for the ability of SC3 to reduce the surface tension of the medium. Yet, it may very well be that other cell-wall proteins are indispensable for formation of aerial hyphae. In the maize pathogen *Ustilago maydis* a class of structural cell-wall proteins was identified that has a role in the formation of aerial hyphae (Wösten *et al*., 1996). Disruption of the *Rep1* gene, which, by processing, produces 12 peptides of 35–53 aa called repellents, resulted in a strain that could not form aerial hyphae and had a wettable phenotype. This phenotype is very similar to that of a *Sch. commune* strain with a disrupted *SC3* gene. Till now the function of repellents was not known.

Structural peptides involved in formation of aerial hyphae by filamentous bacteria

The role of amphipathic peptides in the emergence of aerial hyphae of filamentous bacteria has been best studied in *Streptomyces coelicolor* and *Streptomyces tendae*. In both species, 'bald' (*bld*) mutants have been isolated that are unable to form aerial hyphae under certain growth conditions. This was found to be correlated with the failure to secrete SapB and streptofactin, respectively (Willey *et al*., 1991; Richter *et al*., 1998). SapB consists of 18 amino acids (Willey *et al*., 1991), while streptofactin is an octapeptide (Richter *et al*., 1998). Both peptides bear a nonproteinaceous moiety and are believed to be synthesized non-ribosomally like the peptide antibiotics (Richter *et al*., 1998; Willey *et al*., 1993). Streptofactin and SapB are surface active. They reduce the water surface tension to as low as 39 mJ m^{-2} and 32 mJ m^{-2}, respectively (Richter *et al*., 1998; Tillotson *et al*., 1998). Like SC3 of *Sch. commune*, both SapB and streptofactin are believed to facilitate the emergence of aerial hyphae by breaking the surface tension at the colony–air interface (Willey *et al*., 1991; Richter *et al*., 1998). The reduction in water surface tension by these peptides is at least enough to enable hyphae of *Sch. commune* to breach the water–air interface (see above).

The functional homology of SapB and streptofactin is demonstrated by their ability to restore to *bld* mutants of either *Str. coelicolor* or *Str. tendae* the capacity to form aerial hyphae upon the exogenous addition of either peptide. Surprisingly, the structurally unrelated fungal SC3 hydrophobin also extracellularly complements *bld* mutants of both *Streptomyces* species (Tillotson *et al*., 1998), while the application of streptofactin to the ΔSC3 strain of *Sch. commune* restores its ability to form aerial hyphae (Wösten *et al*., 1999b; see above). Whereas the vegetative (i.e. the substrate) hyphae formed by the *bld* mutants are hydrophilic, the aerial hyphae of extracellularly complemented *bld* mutants, like that of wild-type aerial hyphae and spores, are hydrophobic (Richter *et al*., 1998). More strikingly, unlike wild-type streptomycete aerial hyphae that curl and septate into spores, aerial hyphae formed by *bld* mutants after the addition of SapB, streptofactin or SC3 to the colony surface do not curl nor septate to form chains of spores (Tillotson *et al*., 1998). From this it is concluded that the addition of amphipathic peptides or proteins does not restore the capacity of the mutants to undergo complete morphological differentiation, rather it allows the release of vegetative hyphae from the colony surface. Their upward growth is responsible for the white, fuzzy appearance of the colony.

Similar to the situation in *Sch. commune*, not all surface-active molecules induce the formation of aerial hyphae (Richter *et al*., 1998). Viscosin of *Pseudomonas fluorescens* (Neu *et al*., 1990) and fengycin and surfactin of *Bacillus subtilus* (Vanittanakom *et al*., 1986; Kakinuma & Arima, 1969) are all shown to be ineffective. These data show that only specific surface-active molecules can restore aerial hyphae formation in filamentous micro-organisms and that the structurally unrelated molecules found in filamentous prokaryotic and eukaryotic microbes have been functionally converged.

It is not yet clear whether SapB and streptofactin have a role once the filaments of *Str. coelicolor* and *Str. tendae*

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Fig. 2. Immunolocalization of SapB in a 5-d-old colony of *Str. coelicolor* grown on soy–mannitol medium at 30 °C. Aerial (a) and submerged hyphae (b) are shown. Fixation, embedding and immunolabelling of cultures were performed as described by Wösten *et al.* (1994a) except with the modification that Unicryl was substituted for K4M resin. Polyclonal antibodies raised against SapB (Willey *et al.*, 1991) were purified with an acetone-powder of mycelium of a shaken culture of the *bldA* mutant of *Str. coelicolor* and diluted 1000 times. Similar results were obtained when freeze substitution instead of convential fixation was used or when *Str. lividans* instead of *Str. coelicolor* was labelled. No signals were observed in standing cultures of the *bld261* mutant or in liquid shaken cultures of *Str. coelicolor*. Bar, 300 nm.

have escaped the hydrophilic environment. It had been suggested that SapB might coat aerial structures of *Str. coelicolor*, making them hydrophobic (Willey *et al.*, 1991). However, using immunogold labelling we were unable to localize this peptide either within the aerial hyphae or on their surface. A strong signal was found only in the culture medium (Fig. 2). A similar result was obtained in *Streptomyces lividans*, which is closely related to *Str. coelicolor* (data not shown). From these data we postulate that SapB reduces the surface tension of the medium but that other molecules coat aerial hyphae of *Str. coelicolor* to make them hydrophobic. Like the hydrophobin coating found on fungal aerial hyphae, the coating found on the aerial hyphae of streptomycetes is characterized by a rodlet layer (Wildermuth *et al.*, 1972). Bradley & Ritzi (1968) suggested that the *Streptomyces venezuelae* rodlet mosaic is lipid-like, while Smucker & Pfister (1978) proposed that the rodlets of *Str. coelicolor* are composed of polymers of *N*-acetylated glucosamine. Yet, a significant amount of protein was shown to be present in the sample. However, the partial genome sequence of *Str. coelicolor* has not revealed protein sequences similar to those of the fungal hydrophobins.

Regulation of formation of aerial hyphae in *Str. coelicolor*

Because SapB is diffusable, it is not surprising that *Str. coelicolor bld* mutants grown near a SapB-producing wild-type strain regain the capacity to erect aerial hyphae. Such hyphae are confined to the zone of SapB diffusion from the wild-type (Willey *et al.*, 1991). However, an unexpected observation is that the formation of aerial hyphae can also be restored by growing certain pairs of *bld* mutants in close proximity to each other, even though all these mutants are blocked in SapB biosynthesis. Extracellular complementation is initially unidirectional; one mutant acting as a donor, the other as an acceptor (Willey *et al.*, 1993). Such extracellular complementation assays reveal a hierarchical set of complementation groups in which each mutant can rescue morphogenesis in the mutant strain to the left: $bldJ < bldK < bldA$, $bldH < bldG < bldC < bldD$ (Willey

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et al., 1993; Nodwell *et al*., 1996). Thus, *bldJ* (formerly referred to as *bld261*) can be rescued by all other *bld* mutants, while *bldD* can only be rescued by the wild-type strain. As expected from a complementation assay, once the acceptor is sufficiently complemented by the donor (i.e. sufficiently differentiated), it can 'back-complement' the donor strain, which ultimately leads to full differentiation of both *bld* mutants. The complementation tests suggest the existence of a total of at least five *bld*-dependent signalling molecules that are involved in morphological differentiation. Each signal would trigger the synthesis and release of the next signal, ultimately resulting in the *bldD*-dependent production of morphogenetic molecules like SapB (Willey *et al*., 1993; Nodwell *et al*., 1996). It was hypothesized that this cascade allows the bacterium to couple morphological differentiation with environmental cues like the nutritional status (Champness, 1988; Merrick, 1976; Pope *et al*., 1996) and cell density (Nodwell *et al*., 1996).

Although not all *bld* mutants fit neatly into the hierarchical cascade, the existence of a signalling cascade in *Str. coelicolor* is supported by genetic and biochemical data. The *bldK* locus encodes a putative oligopeptide permease of the ATP-binding cassette (ABC) membrane-spanning transporter family and *bldK* mutants are resistant to the toxic tripeptide bialaphos (Nodwell *et al*., 1996), indicating that the product of the *bldK* locus transports an extracellular peptide. It is hypothesized that the production of this peptide is under control of the *bldJ* gene. Partial purification of the BldK signalling molecule suggests that it is a 655 Da peptide (Nodwell & Losick, 1998). The model predicts that in the wild-type, import of this molecule by the BldK peptide permease induces production of a second extracellular signal that depends on the products of both *bldA* and *bldH*. This signal is secreted and triggers release of the next signal and so on until *bldD* is triggered to release its signal which either directly or indirectly results in SapB production (Nodwell *et al*., 1996). Whether SapB is produced by all growing submerged hyphae, like SC3 in *Sch. commune* (see below), is not known. Not all *bld* genes are directly involved in signalling molecule, transporter or receptor production. For instance, *bldA* encodes a leucyl-tRNA that is required for the translation of the rare UUA-containing mRNA (Lawlor *et al*., 1987).

Most recently, the production of an extracellular regulatory signal(s) from the *Str. coelicolor bldF* mutant strain 166 (Passantino *et al*., 1991) has been studied. The signal, which is heat stable, protease resistant and capable of passing through a dialysis tubing with a molecular mass cut-off of 3 kDa, is present on solid as well as in liquid complex medium (unlike other *bld* mutants). In contrast to the aerial hyphae formed upon the addition of amphipathic peptides, dilution of rich agar medium with strain 166-conditioned liquid medium restored the capacity of both *bldK* (strain NS40; Nodwell *et al*., 1996) and *bldJ* (strain HU261; Willey *et al*., 1993) mutants to undergo complete morphological differentiation (i.e. production of sporulating aerial hyphae) and to produce SapB as well as pigmented antibiotics (Fig. 3). Furthermore, the 166 spent medium also influenced the rate of differentiation in the wild-type, such that a 1:5 dilution (strain 166 spent medium:fresh culture medium) accelerated morphological differentiation in the wild-type strain *Str. coelicolor* J1501 by about 24 h. Presumably, wild-type *Str. coelicolor* is capable of producing the same extracellular signals as the *bld* mutants but signal synthesis is temporally regulated. The introduction of the signal(s) into the medium during vegetative growth would then be expected to initiate the reprogramming and acceleration of the developmental cycle. The capacity of strain 166 spent medium to accelerate morphogenesis is reminiscent of experiments in which it was shown that homoserine lactone-containing spent medium can elicit precocious expression of cell-density-dependent genes (for a review see Fuqua *et al*., 1996).

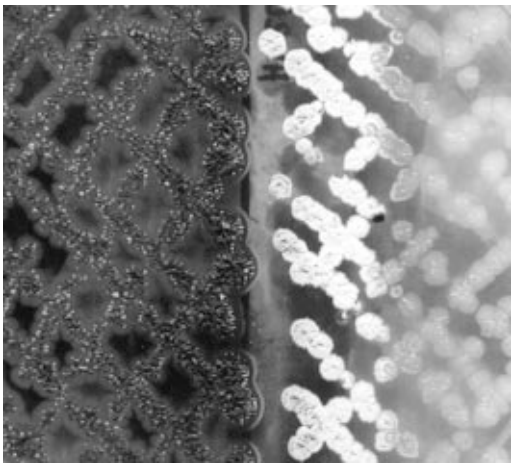

Fig. 3. Extracellular complementation of *bldJ* and *bldF*. *Str. coelicolor* 166 (*bldF*) was plated on the left half of an R2Ye plate (red-pigmented cells), while strain Hu241 (*bldJ*) was plated on the right half. After 4 d growth at 30 °C, colonies of *bldJ* closest to those of *bldF* displayed abundant aerial hyphae formation.

Regulation of aerial hyphae formation in *Sch. commune*

The *SC3* hydrophobin gene of *Sch. commune* is expressed only after a few days of submerged growth (see above). Expression of *SC3* was studied in a culture using green fluorescent protein as a reporter. It was shown that most, if not all, submerged growing hyphae contribute to SC3 production in the medium and that this is not restricted to those hyphae that are destined to grow into the air (Lugones *et al*., 1999). It was proposed that transcription of *SC3* begins only after a submerged mycelium has formed that can support growth of individual aerial hyphae and fruiting bodies by supplying nutrients (Wessels, 1992). How the mycelium senses that it has reached its 'critical mass' remains to be established.

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The *thn* mutant of *Sch. commune* could be considered a phenotypic analogue of the *bld* mutants of *Streptomyces*. It does not produce aerial structures and does not express the *SC3* gene (Wessels *et al.*, 1991). Because a mutation in the *THN* gene has pleiotropic effects it is possible that it is a regulatory gene (Wessels *et al.*, 1991). It was proposed (Wessels *et al.*, 1995) that repression of *THN* gene expression in juvenile cultures prevents the premature transcription of genes involved in emergent growth. This would thus allow formation of an assimilative mycelium that could sustain the growth of emergent structures. Possibly, the *THN* gene is part of a signalling cascade analogous to that proposed in *Str. coelicolor*. Evidence exists that the filamentous fungus *Histoplasma capsulatum* can sense its own numbers (Strauss, 1999), while it was suggested that sensing cell density is involved in differentiation of aerial hyphae in *Aspergillus nidulans* (Lee & Adams, 1994). In *fluG*$^-$ deletion mutants, aerial hyphae do not differentiate to form asexual spores. The deletion could be rescued by growing the mutant next to a wild-type strain, as was done in the case of *bld* mutants of *Str. coelicolor*. It was suggested that *FluG* is involved in the production of an extracellular signalling molecule that above a certain concentration would trigger differentiation of aerial hyphae. The signalling cascades in filamentous fungi may not necessarily be as complex as that of *Str. coelicolor*. In the fungus *Saccharomyces cerevisiae*, a single kind of pheromone can induce an entire developmental process and this development is coupled to environmental signals such as the nutritional status (Herskowiz, 1989).

Concluding remarks

Fungi and bacteria appear to have independently evolved the ability to form aerial structures that allow efficient spore dispersal. Both groups of microorganisms are confronted with the high water surface tension and the need for a submerged feeding mycelium. The former requires molecules that lower the surface tension, the latter monitoring of cell density. As long as the surface-activity is attained by self-assembly and is thereby not toxic to the cell, any such molecule should enable microbial hyphae to escape the aqueous environment to grow into the air. In contrast, monitoring cell density requires specific molecules.

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