The SC15 protein of *Schizophyllum commune* mediates formation of aerial hyphae and attachment in the absence of the SC3 hydrophobin

L. G. Lugones,1,2* J. F. de Jong,2 O. M. H. de Vries,1 R. Jalving,1 J. Dijkstraheus3 and H. A. B. Wösten2

1Groningen Biomolecular Sciences and Biotechnology Institute (GBB), Department of Molecular Plant Biology, University of Groningen, Kerklaan 30, 9751 NN Haren, the Netherlands.
2Microbiology, Institute of Biomembranes, University of Utrecht, Padualaan 8, 3584 CH Utrecht, the Netherlands.
3Centraalbureau voor Schimmelcultures, Uppsalalaan 8, 3584 CT Utrecht, the Netherlands.

Summary

Disruption of the SC3 gene in the basidiomycete *Schizophyllum commune* affected not only formation of aerial hyphae but also attachment to hydrophobic surfaces. However, these processes were not completely abolished, indicating involvement of other molecules. We here show that the SC15 protein mediates formation of aerial hyphae and attachment in the absence of SC3. SC15 is a secreted protein of 191 aa with a hydrophilic N-terminal half and a highly hydrophobic C-terminal half. It is not a hydrophobin as it lacks the eight conserved cysteine residues found in these proteins. Besides being secreted into the medium, SC15 was localized in the cell wall and the mucilage that binds aerial hyphae together. In a strain in which the SC15 gene was deleted (ΔSC15) formation of aerial hyphae and attachment were not affected. However, these processes were almost completely abolished when the SC15 gene was deleted in the ΔSC3 background. The absence of aerial hyphae in the ΔSC3ΔSC15 strain can be explained by the inability of the strain to lower the water surface tension and to make aerial hyphae hydrophobic.

Introduction

After forming a submerged feeding mycelium, filamentous fungi may leave the aqueous environment to form aerial (reproductive) structures. Formation of aerial structures has been described as a two-step process (Wösten et al., 1999). In the first step surface tension of the moist environment is lowered to enable hyphae to breach the medium–air interface. In the second step aerial hyphae become hydrophobic. In the basidiomycete *Schizophyllum commune* both processes are mediated by the Class I hydrophobin SC3 (Wösten et al., 1993; 1994a; 1999). Hydrophobins are small-secreted proteins (about 100 aa) that generally occur in the ascomycetes and the basidiomycetes. Two classes of hydrophobins are distinguished, Class I and Class II (Wessels, 1994). Both are characterized by eight conserved cysteine residues and a typical hydropathy pattern (Wösten and Wessels, 1997; Wessels, 1997). These proteins function by self-assembly at hydrophilic hydrophobic interfaces forming membranes which are highly insoluble in the case of the Class I hydrophobins (Wösten et al., 1993; 1994a; 1995). Disruption of the SC3 gene in *S. commune* affected not only formation of aerial hyphae but also attachment to hydrophobic surfaces (Wösten et al., 1994b; van Wetter et al., 1996). However, these processes were not completely abolished, suggesting that other molecules were involved. Two other monokaryotic strains have been identified that do not produce SC3, i.e. the MATB<sup>thin</sup> and thin mutant strains (Wessels et al., 1991; Ågeirsdóttir et al., 1995). The former strain has a mutation in one of the genes in the MATB mating type locus, leading to a constitutive expression of the MATB-on phenotype. As a result, a mycelium is formed that does not produce aerial hyphae. The thin strain also forms a flat mycelium and is mutated in a gene that is putatively involved in G-protein mediated signal transduction (Fowler and Mitton, 2000).

SC3 can be assembled in the culture medium of a monokaryon or a dikaryon by sparging the medium with air. The highly abundant SC15 protein was shown to co-precipitate with the highly insoluble assemblages of SC3 upon centrifugation (Wösten et al., 1993). After treatment with trifluoroacetic acid (TFA) SC3 was soluble in 60% ethanol whereas SC15 remained insoluble (Wösten et al., 1993). We here report the cloning of the SC15 gene and show that SC15 is responsible for formation of aerial hyphae and attachment in the absence of the SC3 hydrophobin.
Results

Characterization of the SC15 protein

Aggregated proteins that result from bubbling air through growth medium of 5-day-old static cultures of the wild-type monokaryons 4–40 and 4–39 were analysed by SDS PAGE. Bands at 24 kDa as well as 17 and 15 kDa were observed (Fig. 1, lane 1). The former band represents the SC3 hydrophobin, whereas the protein at the 17 kDa band was dubbed SC15. Antibodies raised against the 17 kDa protein also reacted with the 15 kDa protein. Both bands were absent in media of a ΔSC15 strain and the N-terminal sequence of the 15 kDa form was found in the deduced amino acid sequence of SC15 (see below). These findings confirm that both the 17 and the 15 kDa bands represent forms of SC15. The SC15 bands were also observed in growth media of static cultures of the ΔSC3 strain 72–3 and the dikaryon that resulted from mating the wild-type strains 4–40 and 4–39 (not shown). In contrast to the monokaryons, levels of both bands were highly variable in the dikaryon. Their presence positively correlated with the presence of SC3. The SC15 forms were absent in static cultures of the MATB con strain (not shown) and the thn strain (Fig. 1, lane 3). In the case of strain thn a 16 kDa protein was observed that did not react with the SC15 antiserum. It is probably an unrelated protein. In shaking cultures of the wild-type strain 4–39 the SC15 bands running at 15 and 17 kDa were faint or absent (Fig. 1, lane 2) but a band at 12 kDa reacted with the SC15 antiserum. Its N-terminal sequence showed that it is a proteolytic degradation product of SC15 (see below).

As SC15 co-precipitated with assembled SC3 upon centrifugation of culture medium that had been sparged with air, the question arose whether the aggregation of the former depended on the assembly of the latter. To examine this, growth media of the ΔSC3 strain 72–3 and the wild-type strain 4–39 were centrifuged for 30 min at 6000 g in order to remove insoluble material, after which SC15 was isolated by bubbling air through the supernatants. For both strains SDS-PAGE of the aggregated protein revealed proteins of 15 and 17 kDa that reacted with the SC15 antibody (not shown). From this experiment it was concluded that SC15 aggregates at the water–air interface independently of SC3.

Aggregated SC15 was insoluble in water, even after treatment with TFA, but unlike assembled SC3 it could be directly dissolved in 2% SDS or 100% acetic acid. After TFA treatment both proteins were soluble in acetic acid. By dialysis against water, SC15 in acetic acid could be rendered soluble in water, after which it could be precipitated again by vortexing air into the solution. SC15 dried down from a water solution on a grid did not show an apparent ultrastructure after surface shadowing (not shown). In contrast, SC3 assembled into the previously observed mosaic of parallel rodlets (Wösten et al., 1993).

Isolation of the SC15 gene

N-terminal sequencing of the intact 17 kDa protein was unsuccessful. Therefore, internal fragments obtained by proteolysis as well as the 15 and 12 kDa forms found in the medium were subjected to N-terminal sequencing. One of the proteolytic fragments of the 17 kDa form yielded the sequence: ATGSILPQLETVADSG. In order to isolate the cDNA encoding SC15, a degenerate primer was synthesized based on the amino acid residues 5–12 of the internal amino acid sequence taking into account the codon usage of six genes from S. commune (SC3; SC7; SC14; SC4; SC1 and GPD, Schuren, 1992). RT-PCR with this primer and an oligo dT26 using total RNA of S. commune wild-type strain 4–40 yielded a 600 bp band. RACE-PCR on total RNA was used to obtain the 5′ end of the mRNA. A band of 380 bp was amplified. The complete cDNA was predicted to encode a protein of 191 aa (Mw 19 973) flanked by a 5′-non-translated region of 63 bp and a 3′ non-translated region of 171 bp. The coding sequence starts with a putative signal sequence for
secretion of 22 aa (PSIGNAL, PC/GENE package). The predicted mature protein (Mw 17 392) has a high content of Leu (21%), Ala (13%), Gly (10%) and Val (8%) and has a pl of 4.19. Because SC15 does not contain cysteine residues it is not a hydrophobin. As shown in Fig. 2 the mature protein appears moderately hydrophilic at its amino terminal half (average hydrophobicity –0.54) but highly hydrophobic at the carboxy-terminal half (average hydrophobicity 1.6). N-terminal sequencing of the 15 and 12 kDa forms of SC15 showed that these derivatives have N-terminal truncations of 37 and 70 amino acids, respectively. Thus, these forms lack about half and almost all of the hydrophilic N-terminal region.

A search for homologous sequences in the database resulted in a fruiting body specific cDNA (FVFD16) from *Flammulina velutipes* but no other homologues were found. FVFD16 showed homology to the hydrophilic N-terminal part of mature SC15. In a stretch of 61 amino acids 36% of the residues were identical and 18% were conserved substitutions. Strikingly, a shift in reading frame at position 384 of the FVFD16 cDNA (frame 3–2) resulted in a stretch of amino acids with homology to the hydrophobic C-terminal part of the SC15 protein (29% identical and 32% conserved residues). Taking this frame shift into account, the protein shows a similar hydropathy pattern (Fig. 2B).

The RT-PCR fragment of SC15 was used to screen a genomic cosmid library of wild-type strain 4–40. A positive clone was found and a 4.8 kb *Sal* fragment was subcloned which contained the whole SC15 gene. By comparing the cDNA and the genomic sequence (GenBank accession: AJ007503) three short introns of about 50 base pairs were found with splice sites and internal branch sites conforming to the consensus sequences for introns of filamentous fungi (Gurr *et al*., 1987; Lugones *et al*., 1999). A TATA box could be found 12 bp upstream of the transcription starting point that was part of a 15 bp fragment also found in the SC3 gene (only one base difference).

**Expression of SC15**

Total RNA was isolated from 7-day-old static cultures of the wild-type monokaryon 4–39, its derivative the ΔSC3 strain 72–3, the wild-type dikaryon 4–39 ¥ 4–40 and the mutant strains MATB<sup>con</sup> and thn. The RNA was hybridized to the SC15 coding sequence (Fig. 3). SC15 mRNA accumulation was high in the monokaryons 4–39 (wild...

![Fig. 2. Alignment (upper panel) and hydropathy plot of SC15 (191 aa) and FVFD16 (GenBank accession: D88441; 178 aa assuming a frame shift at position 384 as indicated by the arrow in both panels, see Results for details). The hydropathy values were calculated using a window size of 5 amino acids (Kyte and Doolittle, 1982).](image-url)
type) and 72–3 (ΔSC3 strain). It was also detected in the dikaryon but to a lesser extent. SC15 was not expressed in the MATB<sup>con</sup> and then strains, as was the case for SC3 (Fig. 3). This suggests that both genes are similarly regulated.

**Immunolocalization of SC15**

Immunogold labelling was used to localize SC15 in the mycelium of wild-type strains 4–39 and 4–40. The protein was mainly found in the mucilage that binds aerial hyphae together. Some labelling was also observed in cell walls of aerial hyphae. In contrast to SC15, SC3 was found to have accumulated at surfaces in contact with air (Fig. 4). Both proteins were not localized in cell walls of submerged hyphae (not shown).

**The role of SC15 in the formation of aerial hyphae**

The monokaryotic wild-type strain 4–40 was transformed with the SC15 deletion construct pSC15D. Three out of 40 phleomycin-resistant colonies did not produce SC15 as was determined by screening with the SC15 antiserum. Of these transformants, one was found to have a then mutation. Southern analysis showed that the SC15 gene was disrupted in the other two colonies (data not shown). Chromosomal DNA digested with SalI was hybridized to the RT PCR product (see above). In the case of the wild-type, a strong signal was observed at 4.8 kb. The ΔSC15 strains showed a faint signal at 5.5 kb explained by the fact that only a 129 bp fragment could hybridize with the probe.

Formation of aerial hyphae was not affected in the ΔSC15 strain (Fig. 5A). Colonies on solid medium had a white fluffy appearance similar to that of the wild-type strain. Scanning electron microscopy showed that the number of individual aerial hyphae and hyphae in bundles was not changed compared to the wild type (not shown). To exclude that the absence of the SC15 protein in the ΔSC15 strain was compensated by increased amounts of SC3, production of these proteins was determined in culture media of the wild-type and ΔSC15 strains. This showed that deletion of SC15 did not affect production of SC3 (not shown).

A ΔSC3ΔSC15 strain was obtained by crossing the ΔSC15 strain with the ΔSC3 strain. Progeny of the cross were screened for SC3 and SC15 secretion using immunodetection. Approximately 25% of the spores did neither
secrete SC3 nor SC15, showing that these genes are not linked. Disruption of both genes in one of the strains was confirmed by Southern hybridization. Deletion of SC15 clearly affected formation of aerial hyphae in the ΔSC3 background. Analysis of 50 spores derived from the crossing ΔSC15ΔSC3 × ΔSC3 showed that the strongly reduced ability to form aerial hyphae segregated with the inability to secrete SC15. Colonies of the ΔSC3ΔSC15 strain had a greyish rather than a white appearance (Fig. 5A), explained by the fact that aerial hyphae only formed in patches (Fig. 5B). In these patches the number of individual aerial hyphae and hyphae in bundles was similar to that of the aerial mycelium of the ΔSC3 strain (Fig. 5D).

As reported earlier (Van Wetter et al., 1996) formation of aerial hyphae and surface hydrophobicity were decreased but not abolished in the ΔSC3 strain. Water droplets with high contact angle were stably maintained at the surface of wild-type colonies but were absorbed after a few minutes in the case of the ΔSC3 strain. Surface hydrophobicity of the ΔSC15 strain was similar to that of the wild type. In contrast, the ΔSC3ΔSC15 strain was completely wettable, i.e. the water droplet was immediately absorbed by the mycelium. Surface wettability was assessed in more detail by analysing holes in the mycelium made by a needle (Fig. 6). Cryo SEM showed progressively larger areas of wetted mycelium around the holes of 5 day old cultures of wild type < ΔSC15 < ΔSC3 < ΔSC3ΔSC15. In contrast to the differences between wild-type and ΔSC15, those between wild type and ΔSC3 and between the ΔSC3ΔSC15 and the other strains were significant (P ≤ 0.05).

Strains were also grown in static liquid cultures. After the liquid medium was colonized, wild-type and ΔSC15 strains formed abundant aerial hyphae. In contrast, aerial hyphae formation in the ΔSC3 strain was reduced, whereas it was almost completely abolished in ΔSC3ΔSC15 strain (Fig. 5C). Previously, it was reported that aerial hyphae formation in the ΔSC3 strain was decreased compared to the wild-type strain as a result of the reduced capacity to lower the surface tension of the medium (Wösten et al., 1999). Indeed, 5-day-old culture media of the wild-type strain and the ΔSC3 strain decreased the surface tension from 72 mJ m⁻² to 44 ± 5 and 52 ± 5 mJ m⁻², respectively, in a 4 h period. The ΔSC15 strain reduced the water surface tension similar to that of the wild-type strain (40 ± 3). In contrast, the culture medium of the ΔSC15ΔSC3 strain was not surface active (65 ± 2). From this we conclude that absence of aerial hyphae in the latter strain is due to the inability to reduce the water surface tension. Pendant droplets of the culture media of the ΔSC3 and ΔSC15ΔSC3 strains adsorbed at the tip of a pipette could easily be sucked up in the pipette after surface tension measurements. In contrast, a discrete hydrophobin film had formed at the medium air interface in the case of the wild-type and the SC15 strain. This film hampered sucking up of the pendant droplets (Fig. 7). From this it is concluded that, in contrast to SC3, SC15

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The role of SC15 in attachment to hydrophobic surfaces

Wild-type, ΔSC3, ΔSC15 and ΔSC3ΔSC15 strains were grown over a Teflon surface to determine attachment to this hydrophobic solid. The wild-type and the ΔSC15 colonies remained attached to the Teflon after stripping the solid with 3% agarose. Hyphae of the ΔSC3 strain detached at 1% agarose, whereas those of the ΔSC3ΔSC15 colony were already removed by 0.3% agarose. Thus, SC15 is involved in attachment to hydrophobic surfaces in the absence of SC3.

Discussion

Formation of fruiting bodies (e.g. brackets and mushrooms) by homobasidiomycetes is a very complex but poorly understood developmental process. A role for the SC7 and SC14 genes of S. commune (Schuren et al., 1993a), the galectin genes of Coprinus cinereus (Cooper et al., 1997) and pri3 of Agrocybe aegerita (Sirand-Pugnet and Labarrère, 2002) has been proposed on basis of their expression. However, hydrophobins are so far the only secreted proteins that by gene deletions have been shown to be instrumental in formation of fruiting bodies (van Wetter et al., 2000a). A first step in fruiting body development is the aggregation of aerial hyphae. These hyphae bundle by embedding themselves in mucilage. Monokaryons of S. commune do not fruit, yet, they form aerial hyphae that may also form bundles. Formation of aerial structures in the monokaryon could thus provide a good system to understand the first stages of fruiting body development. Indeed, after having established the role of SC3 in the formation of a hydrophobic layer on aerial hyphae, a function for SC4 in the fruiting body could be envisioned. Apart from aerial growth, hyphal attachment to hydrophobic surfaces can be studied with monokaryons of S. commune. It has been shown to be a good model system for interaction of fungi with hydrophobic surfaces, e.g. those of wood or hosts such as plants and animals (Wösten et al., 1994b). The SC3 hydrophobin was shown to be involved in both aerial hyphae formation and in attachment. We here describe the SC15 protein that has similar functions in S. commune under conditions that SC3 is not produced. SC15 may also have a structural role in the mucilage that binds aerial hyphae together.

SC3 and SC15 are the most abundantly secreted proteins in the growth media of the coisogenic S. commune monokaryotic laboratory strains 4–40 and 4–39 (Wösten et al., 1993). An involvement of SC15 in formation of aerial hyphae was suspected because mutations that affect formation of aerial hyphae (thn and MatBcon) suppress expression of both SC3 and SC15. Moreover, when SC3 was assembled in the culture medium by introducing air...
bubbles, SC15 aggregated as well (Wösten et al., 1993). This suggested a specific interaction between the two proteins. However, this paper shows aggregation of SC15 in the absence of SC3, making an interaction less likely. An interaction between SC15 and SC3 could also not be shown by incubating hydrophobin coated Teflon in medium containing SC15 (J. F. de Jong and L. G. Lugones, unpublished).

The SC15 gene was cloned based on the N-terminal sequence of an internal fragment. The deduced amino acid sequence showed a signal sequence for secretion. The predicted N-terminal half of the mature protein is moderately hydrophilic, whereas the C-terminal half is highly hydrophobic. The deduced molecular weight of SC15 (17 392) agreed with the mobility of one of the forms of the protein in SDS PAGE. The other forms running at apparent molecular weights of 15 and 12-kDa were shown to be the result of proteolysis in the N-terminal part. They lack approximately half and almost all of the hydrophilic N-terminal part of SC15 respectively. It is not yet known whether these truncated versions are active forms.

Surface tension experiments showed that SC15 is moderately surface active. A ΔSC15 strain reduced the surface tension almost similar to that of the wild-type strain due to the surface activity of SC3. However, lowering of the surface tension was completely abolished in the ΔSC3ΔSC15 strain. The capacity of the ΔSC3 strain to lower the surface tension to 44 mJ/m² (Wösten et al., 1999) can thus be attributed to SC15. This drop in surface tension is sufficient to allow hyphae to breach the water–air interface to grow into the air. Yet, in the presence of SC3, the hydrophobin and not SC15 will be responsible for the lowering of the surface tension of the medium. This is concluded from the fact that a typical hydrophobin membrane was formed at the medium–air interface in the case of the wild-type and the ΔSC15 strains. In contrast, the ΔSC3 and the ΔSC3ΔSC15 strains did not form such as a membrane. This experiment also showed that SC15 does not self-assemble into a discrete protein film.

Immunolocalization showed that SC15 was abundantly present in the mucilage that binds aerial hyphae together. The abundance of the protein suggests that SC15 may have a structural role. Possibly, it could determine the gelating properties of the mucilage. A role for SC15 in determining surface properties of hyphae could only be shown in the absence of SC3. In a wild-type strain SC3 coats individual aerial hyphae or their bundles (Wösten et al., 1993; 1994a), making them highly hydrophobic. Moreover, the hydrophobin coating mediates attachment to hydrophobic solids (Wösten et al., 1994b). The absence of SC15 in the ΔSC15 strain did not affect the coating of SC3 at hyphal surfaces. However, SC15 became exposed at the surface of the ΔSC3 strain, where it could partly substitute for SC3 in making hyphae hydrophobic and in mediating attachment to hydrophobic solids.

SC15 is the second example of a fungal protein that can fulfil functions that are normally attributed to hydrophobins. Formation of aerial hyphae and surface hydrophobicity were severely affected when the Rep1 gene of the maize pathogen Ustilago maydis was deleted. This gene encodes a precursor protein that is spliced in 12 cell wall localized peptides that are collectively known as repellents and that do not share any identity to hydrophobins (Wösten et al., 1996). Surprisingly, when the hydrophobin genes HUM2 (Bohlmann, 1996) and HUM3 (H. J. Deelstra and H. A. B. Wösten, unpublished data) of U. maydis were deleted formation of aerial hyphae and surface hydrophobicity were much less, if at all, affected. This shows that surface proteins have evolved in fungi that can take over the function of hydrophobins, at least under certain conditions.

This paper shows that SC3 and SC15 are co-regulated in our laboratory strains under laboratory conditions. However, recent findings indicate that in nature SC3 and SC15 are not necessarily co-expressed (J. F. de Jong and L. G. Lugones, unpublished). A monosporous isolate from a S. commune fruiting body recently collected from a forest near Wageningen, the Netherlands, formed SC3 and SC15 on minimal medium. However, when grown on birch sawdust only SC15 was produced. Yet, the strain formed aerial hyphae and thus seems to be phenotypically wild-type. Could it be advantageous for the fungus to suppress formation of SC3 under certain conditions while still producing SC15? Previously, it has been shown that SC3 (and other hydrophobins) affect the cell wall composition (van Wetter et al., 2000b). Young cultures not yet expressing SC3 produced more mucilage (i.e. water-soluble glucan) than older cultures that produced the hydrophobin, while the amount of glucan cross-linked to chitin was lower. In the ΔSC3 strain levels of mucilage remained high throughout growth. The fact that SC3 was not expressed in a natural isolate during growth on wood suggests that this strain may form a mucilage on this substrate. Formation of a mucilagelayer encapsulating hyphae during growth on wood has previously been shown for Phanerochaete chrysosporium (Ruel and Joseleau, 1991). Such a mucilage may function as a storage compound, may provide resistance to desiccation and/or provide proper conditions for hydrolytic enzymes. Whereas SC3 seems to prevent mucilage formation, presence of SC15 (i.e. in the ΔSC3 strain) does not interfere with this process. As mentioned above, SC15 may even have a structural role in the extracellular matrix. Our results also indicate that during mucilage formation SC15 could substitute for SC3 in mediating attachment, in lowering the surface tension and in providing surface hydrophobicity.
Although a ΔSC15 dikaryon was able to fructify and sporulate, a role for SC15 in fructification cannot be discarded. The expression of the homologous FVFD16 of *F. velutipes* during initial stages of fructification (Ando et al., 2001) and preliminary results showing that a SC15:GFP translational fusion is expressed by certain hyphae in fruiting bodies (L. G. Lugones, unpublished results) point in this direction. Possibly, it may have a structural function in the mucilage of the fruiting body tissue. These aspects are currently being addressed.

**Experimental procedures**

**Strains, growth conditions and transformation**

The following strains of *Schizophyllum commune* were used: 4–39 (MATA41 MATB41, CBS 341.81), its derivative 72–3 which has a deletion in the SC3 gene (Wösten et al., 1994b; van Wetter et al., 1996), 4–40 (MATA43 MATB43, CBS 340.81) which is co-isogenic to 4–39, a dikaryotic strain resulting from the mating between 4–39 and 4–40, a 4–39 strain with a *thn* mutation (Wessels et al., 1991), and a MAT-B\(^+\) strain (obtained by mating a MATB\(^+\) MATA\(^+\) strain [1758–6] with strain 4–40). The different strains were grown at 25°C in the light or at 30°C in the dark in minimal medium (Dons et al., 1979), either or not solidified with 1.5% agar. Cultures in liquid medium were either or not shaken at 225 r.p.m. Static cultures were obtained in two ways. Three ml of mycelial macerate was spread on the bottom of Petri dishes. After 3 days 40 ml of minimal medium was injected under the mycelial mat. The floating mycelium was allowed to grow for four additional days. Alternatively, homogenates were directly diluted in excess of minimal medium (Wösten et al., 1999).

**Molecular techniques**

RNA was extracted with the hot-phenol procedure from mycelium, which was ground to a powder under liquid nitrogen in a mortar (Wessels et al., 1987). DNA and RNA hybridizations were done according to Schuren et al. (1993b). cDNA was isolated as described (Lugones et al., 1996). For the isolation of a SC15 cDNA the degenerate primer CATATCTCGAGATCCT(C/G)CC(C/G/T)CA(G/A)CT(C/G)GA(G/A)AC(C/G/T)GT was used in combination with a oligo dT26. The missing 5′ end of the SC15 cDNA was amplified by RACE-PCR using the primer TTGCCC TTAGCTCCTC and the oligo dT26 primer. For gene cloning, a cosmids library of strain 4–40 (Giasson et al., 1989) was screened using the incomplete SC15 cDNA as a probe.

**Disruption of the SC15 gene**

Plasmid pSP72EVEI was used to make a disruption construct. This plasmid contains a phleomycin resistance cassette cloned between the *EcoRV* and *EcoRI* sites from pSP72 (Promega) (Schuren and Wessels, 1994). At both sides of the resistance cassette DNA fragments of about 2.5 kb were inserted, encompassing a short part of the SC15 coding region and 5′ upstream sequences and the downstream sequences of SC15 respectively. In the resulting construct pSC15D, a 640 bp *BglII*/*KpnI* fragment of the SC15 gene has been replaced by the 1380 bp phleomycin resistance cassette. As a consequence, only the coding region for 42 N-terminal amino acids (from the 191 that constitute SC15) is contained in the construct. To rule out differences between strains other than the ones caused by the disruption, both ΔSC3 and ΔSC15 strains were crossed back to the wild-type strain 4–39 five times.

**Protein analysis**

SDS-PAGE, protein staining, Western blotting and immunostaining were performed as described in Lugones et al. (1996). Assembled SC3 was dissolved in TFA at 4°C (Wösten et al., 1993). After centrifugation the supernatant was taken to dryness in a stream of nitrogen and SDS sample buffer was added.

**Preparation of antiserum and immunolocalization**

Rabbits were injected five times at 10 day intervals with 300 μg SDS PAGE-purified SC15. The antisera reacted specifically with SC15 on Western blots of total proteins of *S. commune*. Pre-immune serum was unreactive. The antiserum (diluted 1:100) was purified for immunolocalization by incubating it three times for 60 min with cell walls (Sietsma et al., 1977) from the *S. commune* MATB\(^+\) strain at 1 mg (ml antiserum)\(^{-1}\).

For immunolocalization, the wild-type monokaryon 4–39 was grown between two polycarbonate membranes (Wösten et al., 1991), of which the one in contact with the air was perforated with a needle. After four days aerial hyphae grew through the perforations. Both aerial and submerged hyphae (from underneath the upper PC membrane) were collected and fixed with 2.5% formaldehyde, 0.5% glutaraldehyde in phosphate buffer (0.2 M, pH 7.2). The samples were degassed and incubated on ice for 3 h. After washing with water, the samples were dehydrated in an ethanol series, and incubated 16 h in pure Uranyl acetate (Biocell, Cardiff, UK). The samples were then transferred to fresh Uranyl acetate to polymerize for 48 h at −10°C under UV light. Ultrathin sections were blocked with 0.5% BSA in PBS-glycine buffer (6.5 mM Na\(_2\)HPO\(_4\), 1.5 mM NaH\(_2\)PO\(_4\), 2.7 mM KCl, 150 mM NaCl, 20 mM Glycine at pH 7.2) for 15 min. This was followed by an incubation for 16 h with purified antiserum (SC15 1:100) in the same blocking buffer. After washing three times for 10 min with PBS-glycine buffer, sections were incubated with goat-anti-rabbit serum conjugated to 15 nm gold beads (Amersham, 1:20) in blocking buffer for 1 h. After three washes with PBS-glycine and three washes in distilled water (10 min each), sections were contrasted with 1% aqueous uranyl acetate and allowed to dry before examination in a Philips CM 10 electron microscope.

**Wettability of aerial hyphae**

Different strains of *S. commune* were grown on solid medium for 3–5 days at 25°C. Punchtures were made in the
mycelium with a 0.5 mm needle in a zone 1 cm from the periphery of the colony. This part of the colony was excised, quickly frozen in a nitrogen slush and prepared for low-temperature scanning electron microscopy as described by Dijkstra et al. (1991). The amount of water around the puncture was a measure of wettability. The shape of the needle resulted in a compression of the mycelium at one (concave) side of the hole. For this reason, the diameter of the rim of water at the convex side of the hole was measured. Values were Log-transformed and analysed with an unfactorial ANOVA.

Surface activity

The pendant droplet technique was used to determine surface activity. Drops of 18 µl were measured for 4 h with the Krüss Drop Shape Analysis System DSA10 Mk2 according to the instructions of the manufacturer.

Attachment to hydrophobic surfaces

Attachment of hyphae was determined basically as described (Wösten et al., 1994b). Strains were grown on squares of Teflon sheet (FEP; 0.25 mm thick; Norton Fluoroplast, Raamsdonkveer, the Netherlands). To this end, 50 µl of solidified minimal medium (1.5% agar) was placed in the middle of the sheet and inoculated with a cylindrical piece (1.5 mm in diameter; 4 mm in length) of mycelium from the periphery of a fresh grown colony. The mycelium was allowed to grow over the Teflon surface at 25°C under humid conditions. After four days, low melting point agarose (0.3, 0.5, 1, 2 and 3%) was poured over the colony. After solidification of the agarose at room temperature for 15 min the Teflon sheets were stripped from the agarose and examined for the presence or absence of mycelium.

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