Concerted responses between the chitin-binding protein secreting *Streptomyces olivaceoviridis* and *Aspergillus proliferans*

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Streptomycetes belong to the ecologically important bacterial population within soil, which is also inhabited by many fungi. The highly chitinolytic *Streptomyces olivaceoviridis* and the ascomycete *Aspergillus proliferans* were chosen as models to test for interactions among bacteria and fungi. In medium lacking a soluble carbon source, individually cultivated spores of the bacterium *S. olivaceoviridis* and the fungus *A. proliferans* do not germinate. However, as shown by viability tests, cultivation of a mixture of both spore types provokes successive events: (i) stimulation of the germination of *S. olivaceoviridis* spores, (ii) initiation of the outgrowth of some fungal spores to which the *S. olivaceoviridis* chitinase ChiO1 adheres, (iii) massive extension of viable networks of *S. olivaceoviridis* hyphae at the expense of fungal hyphae and (iv) balanced proliferation of closely interacting fungal and *S. olivaceoviridis* hyphae. The replacement of the *S. olivaceoviridis* wild-type strain by a chromosomal disruption mutant (ΔC), lacking production of the extracellular chitin-binding protein CHB1 but still secreting the chitinase ChiO1, provokes (v) germination of each spore type, (vi) retarded development of both partners, followed by (vii) preferential proliferation of the fungus. Together with biochemical and immunomicroscopy studies, the data support the conclusion that CHB1 molecules aggregate to an extracellular matrix, maintaining a close contact, followed by several concerted responses of the bacterium and the fungus.

INTRODUCTION

Streptomycetes efficiently utilize chitin as a sole carbon as well as a nitrogen source (Kutzner, 1981). The features of the highly chitinolytic *Streptomyces olivaceoviridis* have been investigated in detail (Beyer & Diekmann, 1985). This species secretes the chitin-binding protein CHB1 that targets α -chitin, whose linear chains of β -glycosidic linked *N*-acetylglucosamine (NAG) residues are ordered in an antiparallel arrangement (Schnellmann *et al.*, 1994; Zeltins & Schrempf, 1995). The strain possesses a set of chitinases, of which the exochitinase ChiO1 has been analysed in detail (Blaak *et al.*, 1993; Blaak & Schrempf, 1995). The enzyme consists of a catalytic domain, a linker region and a binding domain that can target a range of chitin types, including those within hyphae of fungi. ChiO1 degrades chitin predominantly to *N,N'*-diacetylchitobiose (chitobiose) (Blaak & Schrempf, 1995). This disaccharide, as well as NAG, was identified to be taken up via the newly discovered

ABC transporter Ngc (Xiao *et al.*, 2002). In addition, the strain has a phosphoenolpyruvate-dependent transport system (PTS) for NAG (Wang *et al.*, 2002). Recently the regulation of a corresponding PTS has been explored in *Streptomyces coelicolor* (Rigali *et al.*, 2006). Streptomycetes are Gram-positive bacteria with a highly complex growth cycle. Upon addition of nutrients, spores germinate and the outgrowing tubes extend to long hyphae that form the substrate mycelium. The depletion of nutrients induces the formation of aerial hyphae, in which spores develop. In addition to chitinases, streptomycetes produce many other enzymes (cellulases, amylases, proteases, lipases) that are important for the turnover of macromolecules as well as for humus formation (Kutzner, 1981). The dominant natural habitat of streptomycetes is the soil. Among many other organisms, they encounter here fungi, which play a central role in litter decomposition and biogeochemical cycling (Klein & Paschke, 2004).

The fungal kingdom is very diverse. While plant-associated fungi are often discussed (reviewed by Harrison, 2005), the role of free-living filamentous ones has been little investigated.

Aspergillus species belong to the ascomycetes – filamentous fungi that build an extended network of vegetative hyphae

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Abbreviations: CHB1, chitin-binding protein; *chb1*, gene for chitin-binding protein; ΔC , mutant lacking the *chb1* gene; ChiO1, chitinase; NAG, *N*-acetylglucosamine; *tsr*, gene for thiostrepton resistance; TEM, transmission electron microscopy; WT, wild-type.

and can form spores that are able to withstand harsh environmental conditions. *Aspergillus* hyphae have a complex cell wall which comprises chitin, glucan, mannan and proteins (Adams, 2004).

In this report, we show that in co-cultures *Aspergillus proliferans* and *S. olivaceoviridis* form a closely tangled co-existing network of fungal and streptomycete hyphae. Immunological studies, including a designed *S. olivaceoviridis* mutant strain lacking a functional *chb1* gene for the chitin-binding protein CHB1, revealed the importance of the chitin-binding protein for the close and balanced interaction of the bacterial and the fungal hyphae.

METHODS

Strains. *Streptomyces olivaceoviridis* has been described (Beyer & Diekmann, 1985). The *S. olivaceoviridis* mutant ΔC carries a substitution of the major portion of the *chb1* gene (Schnellmann *et al.*, 1994; Zeltins & Schrempf, 1997) by a thiostrepton resistance gene (*tsr*) and was kindly provided by A. Zeltins from our group. *Aspergillus proliferans* was purchased from the DSM (Braunschweig, Germany). *E. coli* M15[pREP4] (Qiagen) contains the construct pBA1, consisting of the *chb1* gene cloned in the pQ32 vector (Svergun *et al.*, 2000).

Cultivation of strains. The *S. olivaceoviridis* wild-type (WT) and ΔC mutant were grown on agar plates containing complete medium (Schnellmann *et al.*, 1994) until sporulation occurred. *A. proliferans* was grown on agar plates containing yeast extract medium (Vionis *et al.*, 1996) until spores were formed. The spores were removed with sterile water, filtered through cotton, and counted. The spore suspensions of the *S. olivaceoviridis* WT, the ΔC mutant (2.5×10^9 spores ml^{-1}) and *A. proliferans* (2.5×10^8 spores ml^{-1}) were stored at -20°C in 40 % glycerol. Having washed out residual glycerol, spores of each *S. olivaceoviridis* strain (5×10^6) were inoculated separately in a flask (100 ml) containing 5 ml minimal medium (Hopwood *et al.*, 1985) supplemented with 0.5 % NAG, or without carbon source (control). Alternatively, the spores of *A. proliferans* (1×10^6) were mixed with 10-fold excess of those from the *S. olivaceoviridis* WT or the ΔC strain. *A. proliferans* spores inoculated in medium without carbon source served as control. Cultivation was done at 30°C without shaking (to avoid pellet formation). Samples were taken at the indicated time intervals (see Results) and subjected to microscopy. *E. coli* M15[pREP4]/pBA1 (Svergun *et al.*, 2000) was grown in LB medium (Sambrook *et al.*, 1989).

Microscopic investigations and viability tests. Portions of the cultures were inspected under visible light (phase-contrast) at various magnifications. In parallel, the samples were subjected to a viability assay (Fernandez & Sanchez, 2002) and they were subsequently examined under UV using filter sets (Zeiss) for FITC (excitation, HQ 480/40; beam splitter, Q 505 LP; emission, HQ 535/50) and Texas red (HQ 560/55, Q 595 LP, HQ 645/75, respectively). Living cells exhibited green fluorescence, whereas dead mycelia were seen in red.

Immunological studies. Antibodies against CHB1 had been raised previously (Schnellmann *et al.*, 1994). To analyse the presence of CHB1 associated with the fungal hyphae, aliquots of the mycelia obtained during co-culture of *A. proliferans* and *S. olivaceoviridis* WT or ΔC mutant strain were pre-treated with 2 % BSA in PBS and incubated with anti-CHB1 antibodies (diluted 1:200) for 60 min. Secondary Alexa Fluor 647-labelled antibodies (diluted 1:200) were

purchased from Molecular Probes. Samples were inspected under both phase-contrast and UV using a filter set (Zeiss) for Cy5 (excitation, HQ 620/60; beam splitter, Q 660 LP; emission, HQ 700/75).

To test the presence of extracellular CHB1, the culture filtrate was precipitated with $(\text{NH}_4)_2\text{SO}_4$ (90 % saturation). Aliquots of the resuspended proteins were separated in 12.5 % polyacrylamide gels containing 0.1 % SDS, transferred onto a PVDF membrane (Pall Europe), and treated with anti-CHB1 antibodies and secondary antibodies, as described earlier (Zeltins & Schrempf, 1997).

Test for the presence of chitinase. Using *p*-nitrophenylchitobiose as substrate, chitinase activity was tested as described earlier (Blaak *et al.*, 1993; Blaak & Schrempf, 1995). To determine the presence of the chitinase ChiO1, 10 μl portions were spotted onto a nylon membrane and tested for immunoreaction with specific antibodies (raised previously in rabbit). To test for associated chitinase, a 50 μl portion of each culture was dropped onto a slide (pre-treated with polylysine: Schnellmann *et al.*, 1994; Zeltins & Schrempf, 1995), and incubated for 1 h in the presence of 2 % BSA in PBS followed by 1.5 h with anti-chitinase antibodies (diluted 1:200 in 2 % BSA in PBS). After 10 washes with PBS, samples were treated for 1 h with 1:300 diluted secondary anti-rabbit antibodies (labelled with Cy5, Molecular Probes). Finally, slides were washed 10 times with PBS and inspected by microscopy under phase-contrast and UV (with the Zeiss filter set for Cy5; see above for specifications).

Aggregation properties of CHB1. *E. coli*[pREP4]/pBA1 (Svergun *et al.*, 2000) was grown in LB medium (Sambrook *et al.*, 1989) and induced in the exponential phase (OD_{600} 0.6) with IPTG (1 mmol ml^{-1}) for 90 min. Cells were harvested and the His-tagged CHB1 protein was released by osmotic shock treatment as described earlier for an *E. coli* strain containing the same vector with the *chbB* gene (Chu *et al.*, 2001). The washed cells were suspended in 30 mmol Tris/HCl ml^{-1} , pH 8, 20 % (w/v) sucrose, incubated on ice for 10 min, sedimented (8000 g, 15 min, 4°C), resuspended in ice-cold MgSO_4 (5 mmol ml^{-1}) for 10 min and centrifuged for 15 min at 4°C . The supernatant was collected, equilibrated to 50 mmol Na_2HPO_4 ml^{-1} , pH 8, 300 mmol NaCl ml^{-1} , 10 mmol imidazole ml^{-1} , and applied to Ni-NTA resin (Qiagen). The protein was subsequently purified by affinity and ion-exchange chromatography and subjected to gel filtration on a Superdex 75 column (HR 10/30, Amersham Biosciences), equilibrated with 20 mmol Tris/HCl ml^{-1} (pH 7.0) containing 0.1 M NaCl. Peak fractions cross-reacting with anti-CHB1 antibodies were separated in 12.5 % polyacrylamide gels containing 0.1 % SDS and stained with Coomassie brilliant blue R250 (Laemmli, 1970).

Electron microscopy. Gold labelling of CHB1 was based on the instructions of the colloidal gold manufacturer (British BioCell International). After overnight dialysis against 2 mmol borax ml^{-1} pH 8.6 (corresponds to the pI value of CHB1), one part of the protein solution (0.1 mg ml^{-1}) was mixed dropwise with 18.75 parts of the colloidal gold (diameter 10 nm) suspension that had been adjusted to pH 8.6 with K_2CO_3 (100 mmol ml^{-1}). Following a 5 min incubation at room temperature, BSA (10 % w/v) was added to the final concentration of 0.9 % and the solution was agitated for 10 min. After centrifugation (25 000 g, 60 min at 4°C) the supernatant containing the unlabelled CHB1 was removed and the pellet comprising gold-labelled CHB1 was washed with 100 mmol Tris/HCl ml^{-1} pH 8.2 buffer containing BSA (1 %) and NaN_3 (0.1 %), centrifuged and suspended in the same buffer. Aliquots (10 μl) were placed on carbon-coated copper grids (300 mesh), treated with uranyl acetate (Leal-Morales *et al.*, 1988), and inspected by transmission electron microscopy (TEM) with an EM9 microscope (Zeiss). For immunodetection, aliquots (10 μl) of freshly isolated CHB1 were placed on the grids. After saturation with PBS pH 8.3 containing BSA (2 %), the grids were incubated with anti-CHB1 antibodies

and, subsequently, with gold-labelled secondary antibodies (Sigma-Aldrich). The grids were contrasted with uranyl acetate and inspected by TEM.

RESULTS

Features of *S. olivaceoviridis* WT and the ΔC mutant

The *S. olivaceoviridis* WT secretes the chitin-binding protein CHB1 in the presence of chitin, which promotes an efficient transcription of the *chb1* gene (Schnellmann *et al.*, 1994). The chromosomal DNA of the designed *S. olivaceoviridis* disruption mutant strain ΔC (see Methods) contains the *tsr* gene in place of the major portion of the *chb1* gene.

The spores of the ΔC mutant and the WT strain germinated equally well in minimal medium containing low-molecular-mass carbon sources (NAG, sucrose, glucose or glycerol). Many short germ tubes outgrew within 4 h and often formed fine networks that rapidly (within 10 h) expanded to build substrate mycelia, as exemplified for the WT strain (Fig. 1a, b) and the ΔC mutant (Fig. 1d, e) growing in minimal medium supplemented with NAG.

In contrast to the *S. olivaceoviridis* strains (Fig. 1a, b, d, e), *A. proliferans* cannot utilize NAG hence its spores cannot germinate (Fig. 1g, h, white arrows). In control medium lacking NAG, none of the *S. olivaceoviridis* spores (Fig. 1c, f, black arrows) nor those of *A. proliferans* (Fig. 1i, white arrow) germinated (data presented for 22 h).

Responses of *S. olivaceoviridis* WT and *A. proliferans* during co-cultivation

For further studies, spores of each *S. olivaceoviridis* strain were co-cultured with those of the fungus in minimal medium without soluble carbon source. The spores of *S. olivaceoviridis* WT germinated within 4 h and the tiny hyphae formed a tangled network (Fig. 2a), closely associating with the clusters of *A. proliferans* spores (white arrow in Fig. 2a). During prolonged incubation (up to 10 h), the network of the *S. olivaceoviridis* WT hyphae extended considerably (Fig. 2b) among large clusters of many fungal spores (white arrow in Fig. 2b).

Extension of the incubation time to 18 h mainly led to further proliferation of the *S. olivaceoviridis* hyphae, which formed a myceliar network (Fig. 3a). Portions of co-cultures

Fig. 1. Growth characteristics of the strains. Spores of *S. olivaceoviridis* WT (a, b, c), the ΔC mutant strain (d, e, f) or *A. proliferans* (g, h, i) were incubated without shaking at 30 °C in minimal medium supplemented with 0.5% NAG for 4 h (a, d, g) or for 10 h (b, e, h). As a control, spores of each strain were incubated in medium without NAG, for up to 22 h. Germination did not occur, even after 22 h of incubation (c, f, i). Spores of *S. olivaceoviridis* (black arrows) and *A. proliferans* (white arrows) are marked. Bars, 25 μ m.

Fig. 2. Spores of *A. proliferans* (1×10^6) were inoculated in 5 ml minimal medium without carbon source together with spores (1×10^7) of *S. olivaceoviridis* WT (a, b) or together with the ΔC mutant (c, d) and kept without shaking at 30 °C. Aliquots were inspected after 4 and 10 h by phase-contrast microscopy. *A. proliferans* spores (white arrows) are marked. Bar, 25 μm .

were additionally subjected to a viability test. The test had previously been established for bacteria but had recently also been applied to streptomyces hyphae (Fernandez & Sanchez, 2002). This procedure involves differential staining of damaged (dead) cells with propidium iodide, which can only enter damaged (dead) cells and subsequently intercalate into the DNA, leading to red fluorescence. Living hyphae can take up the dye SYTO 9, which after its interaction with DNA results in green fluorescence of hyphae under UV. In contrast to the well-growing (Fig. 3a) and viable (green in Fig. 3b) *S. olivaceoviridis* hyphae, those of the fungi (white arrow in Fig. 3a) were developing slowly up to 18 h and they often appeared dead in the viability test (red-appearing fungal filament; white arrow in Fig. 3b). Further co-cultivation (up to 22 h) led to the emergence of a few more outgrowing fungal hyphae and the *S. olivaceoviridis* hyphae continued to be mostly viable (green) and extending on to fungal spores and some dead fungal hyphae (Fig. 3c white arrowhead, 3d). Further incubation (up to 34 h) led to the extended proliferation of *S. olivaceoviridis* as well as some more fungal hyphae (Fig. 3e, white arrowhead). Extended regions of *S. olivaceoviridis* hyphae were viable (green in Fig. 3f) and only a few patches were dead (red in Fig. 3f). In contrast, well-developing *A. proliferans* hyphae (as judged by phase-contrast microscopy) did not exhibit green fluorescence, indicating that they cannot take up the SYTO 9 dye. Consequently, green fluorescence always correlated only with living hyphae of each of the *S. olivaceoviridis* strains.

Fig. 3. Viability test of co-cultures. Spores of *S. olivaceoviridis* WT (or the ΔC mutant) were incubated together with *A. proliferans* in minimal medium without carbon source, as described in Fig. 2. Having been subjected to a viability test (see Methods), samples of each culture were inspected by phase-contrast under white light (a, c, e, g, i, k) and UV (b, d, f, h, j, l), in the presence of a filter set for FITC or Texas red (see Methods for specifications). Under UV living hyphae appear green, dead ones are red. *A. proliferans* spores (white arrows) and hyphae (white arrowheads) are marked. Bars, 100 μm .

Reduced interaction between *A. proliferans* and the *S. olivaceoviridis* ΔC mutant

During early periods (4–10 h) of co-cultivation the *S. olivaceoviridis* ΔC mutant developed slowly on a few fungal spores (white arrows in Fig. 2c and d). After continued incubation (18 h) the hyphae of the ΔC mutant extended (Fig. 3g, i, k). Up to 22 h only a few fungal spores outgrew to hyphae (white arrowheads in Fig. 3g–j). However, after extension of the co-culture time to 34 h the network of the fungal hyphae extended considerably (white arrowheads in Fig. 3k), among residual dead (red) patches and a few interspersed living (green) hyphae from the *S. olivaceoviridis* ΔC mutant (Fig. 3j).

If NAG was added to each type of co-cultures, the growth characteristics of each *S. olivaceoviridis* strain were very similar (up to 22 h), leading to dominant growth of *S. olivaceoviridis* hyphae (of WT or ΔC) compared to the fungus (Fig. 4). The data indicate that the low-molecular-mass carbon source NAG (0.5 %) is used preferentially in the

additional presence of fungal spores, which cannot germinate with NAG as carbon source (Fig. 1g–i).

Deposition of chitin-binding protein CHB1 within co-cultures

Antibodies were used to score the distribution of CHB1 during co-culture of *S. olivaceoviridis* WT and *A. proliferans* (Fig. 5a, b). CHB1 levels were found to correlate with the expansion of the network of *S. olivaceoviridis* hyphae in the presence of fungus and the amount of CHB1 was notably higher within the closely interacting networks. Many patches of the deposited CHB1 were immunodetected, primarily in regions containing well-developed fungal hyphae in close vicinity to the *S. olivaceoviridis* hyphae

Fig. 4. Viability test of co-cultures in the presence of NAG. Spores of *S. olivaceoviridis* WT or ΔC were grown with those of *A. proliferans* for 22 h in minimal medium supplemented with NAG (0.5 %), as described in Fig. 1. Samples were subjected to a viability test (see Methods) and inspected by light (a, d) and fluorescence microscopy (b, e) as described in Fig. 3. The merged pictures a+b and d+e resulted in c and f, respectively. *A. proliferans* hyphae (white arrows) are marked. Bar, 25 μ m.

Fig. 5. Immunodetection of the CHB1 protein within the co-cultures. *S. olivaceoviridis* WT (a–d) or the ΔC mutant (e–h) were grown in minimal medium with NAG (c, d and g, h) or in minimal medium without NAG but as a co-culture with *A. proliferans* (a, b and e, f). Samples were taken after 63 h, treated with primary anti-CHB1 antibodies and secondary Alexa Fluor 647-labelled antibodies, and inspected under UV (b, d, f, h) in the presence of a filter set for Cy5 (see Methods for specifications). Bars, 25 μ m.

(Fig. 5b). As expected, CHB1 was not immunodetectable when the *S. olivaceoviridis* WT strain was replaced by the ΔC strain (Fig. 5e, f) or when each of the *S. olivaceoviridis* strains was cultivated with NAG as a sole carbon source (Fig. 5c, d and g, h).

Aggregation properties of CHB1 molecules

The purified CHB1 was found to form high-molecular-mass aggregates. Gel filtration (Fig. 6a) and subsequent SDS-PAGE analysis (Fig. 6b) after heating (100 °C) revealed a set of differently sized protein bands containing CHB1. Immunological studies (Western blot, not shown) confirmed that these protein bands corresponded to CHB1 monomers, dimers, trimers (traces) and higher oligomers. The aggregation was found to be independent of the treatment with thiol-reducing agents and still occurred in the presence of moderate concentrations of denaturing agents (urea and guanidinium hydrochloride, data not shown), suggesting hydrophobic interactions. The self-aggregation of gold-labelled CHB1 was visualized by

electron microscopy (Fig. 6c). Likewise, patches of highly aggregated CHB1 were immunodetectable with gold-labelled antibodies, in contrast to the control (Fig. 6c).

Synthesis of chitinase in co-cultures

Chitinase activity was barely detectable in the culture filtrate of each type of co-culture in the early phase (up to 18 h) of growth. Immunological studies (dots spotted onto a filter) showed that the previously identified chitinase ChiO1 (Blaak *et al.*, 1993; Blaak & Schrempf, 1995) was present within culture filtrates. Its amount rose slowly during extension of the cultivation time (up to 3 days) in both types of co-cultures. The resolved protein patterns (SDS-PAGE) of 50-fold concentrated (by ammonium sulphate precipitation) supernatant were identical. Very small (about equal) amounts of a protein (about 55 kDa) were immunodetectable with anti-ChiO1 antibodies within the supernatant of each type of co-culture (data not shown). This finding prompted us to test for the association of the enzyme. Immunological studies revealed that the chitinase ChiO1

Fig. 6. Analysis of the aggregation properties of CHB1. (a) The purified CHB1 protein was subjected to gel filtration, resulting in the peak fractions 1, 2 and 3. (b) Fraction 1 (aliquot of 30 μ l) mainly comprised oligomers (O) and traces of the trimer (T) as shown by SDS-PAGE (lane 4). Fraction 2 (10 μ l) contained mainly the dimer (lane 5). In fraction 3 (30 μ l) the monomer (M) dominated (lane 6). As a control, reference proteins were analysed (lane 7). (c) Purified CHB1 was gold-labelled, placed on to a copper grid, and inspected directly by TEM (left). A sample of the purified CHB1 was placed on a copper grid, and treated with anti-CHB1 antibodies and secondary gold-labelled antibodies (centre). A sample that contained no CHB1 served as control (right). Bars, 70 nm.

was deposited on the fungal spores (white arrows in Fig. 7a–d) already during the early phase (18 h) of cultivation. This effect occurred in both types of co-culture to about equal extent.

DISCUSSION

Spores of fungi and streptomycetes represent the dormant stage of their life cycle. Our studies showed that in the absence of nutrients individual spores of *A. proliferans* and the *S. olivaceoviridis* (WT and ΔC , respectively) do not form germ tubes. In contrast, the bacterial and fungal spores germinate when mixed in minimal medium lacking a carbon source. In co-culture the spores of *S. olivaceoviridis* WT (Fig. 2a, b) or the ΔC mutant (Fig. 2c, d) appear to germinate more rapidly than those of *A. proliferans* (white arrows in Fig. 2). This finding is in agreement with the

observation that the culture filtrate from either type of co-culture did not enhance the germination of the fungal or the *S. olivaceoviridis* (WT or ΔC) spores. Knowledge of the molecular mechanism(s) of spore germination in streptomycetes is still scarce. The process is usually observed after exposure to low-molecular-mass nutrients (Kutzner, 1981). In the initial stage unknown signals trigger the influx of water into spores, leading to swelling and subsequently to outgrowth of a germ tube. An *S. coelicolor* mutant with a non-functional gene for adenylate cyclase was found to have a delay in the germ tube emergence (Susstrunk *et al.*, 1998).

RasA, a small GTP-binding protein, is important for carbon source sensing during *Aspergillus nidulans* germination. In addition, the cAMP/PKA pathway is believed to be involved. Thus germ tube formation is delayed in the absence of the adenylate cyclase gene (Fillinger *et al.*, 2002). GasC, a member of the group III G_{α} proteins, appears to control germination but not the carbon sensing in *Penicillium marneffeii* and it has been proposed that GasC signals through the cAMP/PKA pathway (Zuber *et al.*, 2002). Interestingly, serine/threonine and tyrosine kinases, which control cellular responses to environmental stimuli via complex signal transduction networks in eukaryotes, have recently been found in streptomycetes (Umeyama *et al.*, 2002) and their participation in morphogenesis seems likely.

In contrast to *S. olivaceoviridis* WT, the development of the mutant ΔC , which does not secrete the chitin-binding protein CHB1, is considerably retarded during co-culture with *A. proliferans* (Fig. 2). As visualized by immunomicroscopy, CHB1 forms patches within the network of the *S. olivaceoviridis* WT hyphae interwoven with fungal hyphae and residual fungal spores (Fig. 5a, b). The biochemical and electron microscopy studies revealed that even CHB1 alone forms high-molecular-mass aggregates (Fig. 6), probably via hydrophobic interactions.

During further extension of the co-culture time, the *S. olivaceoviridis* WT strain develops a very well-extended and living mycelium. The fungal hyphae also wrap around the individual or bunched *S. olivaceoviridis* hyphae (Fig. 3a, b). As reported earlier, streptomycetes secrete many extracellular hydrolytic enzymes degrading macromolecules such as glucans, chitin and proteins, which are also located in the cell wall of fungi. Interestingly, the chitinase ChiO1 from *S. olivaceoviridis* was primarily found bound to the fungal spores, suggesting its participation in their degradation. Previous studies have shown that the binding domain of this chitinase mediates its adherence to chitin-containing substrates (Vionis *et al.*, 1996). Therefore, it was not surprising that during early steps of co-cultivation the occurrence of rapidly developing *S. olivaceoviridis* hyphae correlated with the decay of emerging fungal hyphae (Fig. 3b, c, d). Previously, it had been shown that *S. olivaceoviridis* has uptake systems for degradation products such as NAG and chitobiose (Wang *et al.*, 2002; Saito & Schrempf, 2004). Ultimately, the *S. olivaceoviridis* WT and

Fig. 7. Detection of chitinase activity. Spores of *S. olivaceoviridis* WT (a, b) or the ΔC mutant (c, d) were co-cultivated with those of *A. proliferans* in minimal medium without carbon source for 18 h. Samples were treated with anti-chitinase (ChiO1) antibodies followed by secondary Cy5-labelled anti-rabbit antibodies and inspected under phase-contrast (a, c) or UV (b, d) in the presence of a filter set for Cy5 (see Methods for specifications). Controls that were done for both types of co-cultures in the absence of secondary antibodies showed the same result; thus they are presented only for *A. proliferans* with the WT strain under phase-contrast (f) and UV (e). *A. proliferans* spores (white arrows) are marked. Bar, 50 μ m.

the fungus co-grow in a balanced and sustainable manner (Fig. 3e).

In co-culture with *A. proliferans* the *S. olivaceoviridis* ΔC mutant shows considerably retarded development (Fig. 2c, d) compared to the WT strain (Fig. 2a, b). After 34 h of co-culture the hyphae of *A. proliferans* develop very well and overgrow (Fig. 3k) the ΔC hyphae, a large portion of which was shown to be no longer alive (Fig. 3l). Thus, the fungal hyphae appear to feed well on the decaying ΔC strain. Interestingly, various *Aspergillus* species produce a range of enzymes (Klein & Paschke, 2004), some of which (e.g. proteases) and antibiotics (β -lactams) could contribute to the damage of the emerging bacterial hyphae.

This study is believed to be the first to demonstrate an intimate interaction between a *Streptomyces* and an *Aspergillus* strain. Deeper insights into the socio-microbiology of streptomycetes and free-living fungi are of great value for applications and are of high ecological importance.

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