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Bacillus subtilis attachment to *Aspergillus niger* hyphae results in mutually altered metabolism

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Summary

Interaction between microbes affects the growth, metabolism and differentiation of members of the microbial community. While direct and indirect competition, like antagonism and nutrient consumption have a negative effect on the interacting members of the population, microbes have also evolved in nature not only to fight, but in some cases to adapt to or support each other, while increasing the fitness of the community. The presence of bacteria and fungi in soil results in various interactions including mutualism. Bacilli attach to the plant root and form complex communities in the rhizosphere. *Bacillus subtilis*, when grown in the presence of *Aspergillus niger*, interacts similarly with the fungus, by attaching and growing on the hyphae. Based on data obtained in a dual transcriptome experiment, we suggest that both fungi and bacteria alter their metabolism during this interaction. Interestingly, the transcription of genes related to the antifungal and putative antibacterial defence mechanism of *B. subtilis* and *A. niger*, respectively, are decreased upon attachment of bacteria to the mycelia. Analysis of the culture supernatant suggests that surfactin production by *B. subtilis* was reduced when the bacterium was co-cultivated with the fungus. Our experiments provide new insights into the interaction between a bacterium and a fungus.

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Introduction

In the environment, microorganisms occur in complex communities and interact with each other and with other higher organisms (plants and animals). Bacterial–fungal interactions (BFI) have an important impact on the microbial fitness in the soil (Nazir *et al.*, 2010). Fungi and bacteria often harbour properties in interdependent consortia that differ from those of their single components (Tarkka *et al.*, 2009). BFI might be achieved via antibiosis, signalling molecules, modulation of physiochemical environment, chemotaxis, cooperative metabolism, protein secretion or even gene transfer (Frey-Klett *et al.*, 2011). BFIs via molecular communication can be achieved without the physical interaction of the partners, but there are increasing number of reports on direct BFIs. The physical interactions can range from complex polymicrobial communities to highly specific associations. These interactions can also include endosymbiotic relationships [e.g. symbiosis between *Geosiphon pyriformis* and *Nostoc puntiforme* (Kluge, 2002), between *Rhizopus* and *Burkholderia* sp. (Partida-Martinez and Hertweck, 2005)] and in several cases bacterial cells actually attach to the fungal hyphae. Both *Pseudomonas aeruginosa* and *Staphylococcus aureus* can colonize the hyphal forms of *Candida albicans* (Hogan and Kolter, 2002; Peters *et al.*, 2010). *Pseudomonas aeruginosa* can produce antifungal compounds, like phenazines (Gibson *et al.*, 2009) and inhibits *C. albicans* biofilm formation (Holcombe *et al.*, 2010; Morales *et al.*, 2010), while *S. aureus* uses its fungal partner to enter the epithelial tissues during mucosal infection (Peters *et al.*, 2012). Studying BFI interaction has also helped to discover otherwise silent secondary metabolite gene clusters coding for antimicrobial

compounds (Scherlach *et al*., 2013). For example, the soil-derived bacterium, *Streptomyces rapamycinicus*, induces cryptic pathways for production of orsellinic acid and meroterpenoids in *Aspergillus nidulans* and *Aspergillus fumigatus* respectively (Nützmann *et al*., 2011; König *et al*., 2013). Activation of a histone acetyltransferase complex in the fungus, from an unknown bacterial signal, has been shown to play an important role in this bacteria-induced natural product formation from *A. nidulans* (Nützmann *et al*., 2011; 2013).

Another example of BFI shows that in the dispersal of the non-motile fungus, *A. fumigatus* is facilitated by the swarming bacterium *Paenibacillus vortex* (Ingham *et al*., 2011). Complex bacterial–fungal biofilms have also been reported in other contexts, such as mycorrhizal root systems (Nurmiaho-Lassila *et al*., 1997) and rice wine production (Kawarai *et al*., 2007). Immunocapturing methods have identified that *Bacillus cereus* was associated with arbuscular mycorrhizal hyphae and *in vitro* studies showed the attachment of *B. cereus*, isolated from the same soil, to the mycorrhizal hyphae of *Glomus dussii* (Arturson and Jansson, 2003).

The filamentous fungus *Aspergillus niger* and the Gram-positive bacterium *Bacillus subtilis* have long been established as industrial microorganisms for the production of enzymes and secondary metabolites. *Aspergillus niger* is capable of high secretion of proteins such as glucoamylase or xylanases (Kwon *et al*., 2012) and organic acids such as citric, gluconic and oxalic acid (Papagianni, 2007). *Bacillus subtilis* is a major workhorse for protein production because of its high growth rate leading to short fermentation cycles and its ability to secrete proteins in extracellular medium (Westers *et al*., 2004; Antelmann *et al*., 2006). Both *A. niger* and *B. subtilis* have been extensively studied and used as model organisms in various molecular biology studies. While both organisms can be isolated from soil samples, it is not known whether they interact in the environment. Here, we have examined whether direct interaction is possible under laboratory conditions between *A. niger* and *B. subtilis. Bacillus subtilis* NCIB3610 (3610) swarms on semisolid surfaces (Kearns and Losick, 2003) and forms robust biofilms both under laboratory conditions (Branda *et al*., 2001; Mhatre *et al*., 2014) and in the rhizosphere (Chen *et al*., 2012; 2013; Beauregard *et al*., 2013). We examined the transcriptional responses of the interacting partners, *A. niger* and *B. subtilis*, during attachment. We found that in addition to metabolism, genes putatively related to antibacterial and antifungal molecule productions were downregulated in *A. niger* and *B. subtilis* respectively. Reduced production of the antifungal compound surfactin by *B. subtilis* suggests an active adaptation to the partner organism or alternatively, surfactin production is inhibited by *A. niger*.

Results

Co-cultivation of A. niger *and* B. subtilis *on agar medium*

Aspergillus niger and *B. subtilis* 3610 were grown together on agar plate as described in Experimental procedures. After 6 days of incubation, a clear mixed population was observed (Fig. 1A). *Aspergillus niger* mycelium

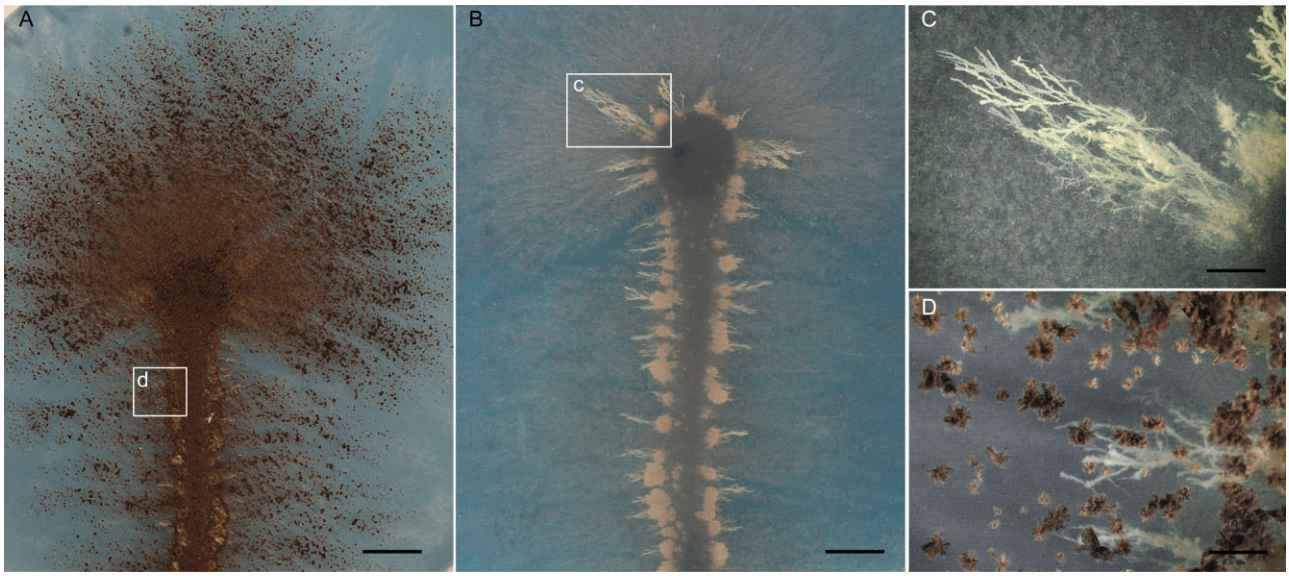

Fig. 1. Co-cultivation of *A. niger* and *B. subtilis* on agar plates.
A. Front side of the agar plate with *A. niger* sporulating colony.
B. Reverse side of the agar plate with visible *B. subtilis* branches along the *A. niger* mycelium. Scale bars for A and B indicate 7 mm.
C and D. Higher magnification on the *B. subtilis* branches. Scale bar for c and d, respectively, indicate 2.5 mm and 1.5 mm.

had colonized the surface, forming aerial hyphae with conidiophores and had also invaded the solid agar medium. The mycelium growing into the agar medium was coated by *B. subtilis* cells and visible as white branches (Fig. 1B and C). No bacterial growth could be identified on the rest of the agar plate. *Bacillus subtilis* was able to colonize the surface in the absence of fungi-forming complex colonies, similar to what was reported previously (Branda *et al.*, 2001). Some parts of the mycelium were not colonized by the bacteria (Fig. 1B). To check the viability of both microorganisms, a cross section of the mycelium including the bacterial patch was restreaked on a fresh agar plate where both microorganisms grew well. Conidiophores were also picked from the aerial structures and inoculated on a fresh agar plate, but no *B. subtilis* colonies were detected suggesting that the bacteria only colonized the submerged parts of the fungal colony in the agar medium.

Co-cultivation of the two microorganisms in liquid-shaken cultures

To get a deeper insight into the interaction between *A. niger* and *B. subtilis* observed on the agar medium, the two microorganisms were mixed in liquid-shaken cultures. Due to growth rate differences, *A. niger* was pregrown for 12 h before *B. subtilis* 3610 was added to the medium. The time-course of bacterial attachment was followed using microscopy (Fig. 2). During the first 2 h, *B. subtilis* single cells had physical contact with the *A. niger* mycelium and started to attach preferentially to the protruding hyphae. Remarkably, not all protruding hyphae were targeted. After 2 h, *B. subtilis* aggregated in a thin cell layer along some hyphae while other hyphae were still free of bacteria. After 4 h, the thin layer of bacterial cells became thicker. Many planktonic cells were still visible and actively moving around in the supernatant, some of these cells were joining the aggregated cells. Between 8 and 24 h, large patches of *B. subtilis* were observed; the cells had changed into roundish shapes, and slightly fewer bacteria were observed in the planktonic population (Movie S1). Interestingly, when the laboratory strain *B. subtilis* 168 was co-inoculated with *A. niger*, no bacterial interaction was observed, suggesting that the interaction is strain specific (Fig. S1). *Bacillus subtilis* 168 is not able to produce surfactin and is swarming deficient, while 3610 has these properties (Kearns and Losick, 2003). Also, when *B. subtilis* 3610 was co-inoculated with *Aspergillus oryzae*, the growth of *B. subtilis* was inhibited both in liquid culture and on agar plates and consistently no attachment was observed (Fig. S2).

To check the strength of bacterial attachment to the hyphae, mycelium containing the attached bacteria after 4 h of co-cultivation was washed with fresh medium and

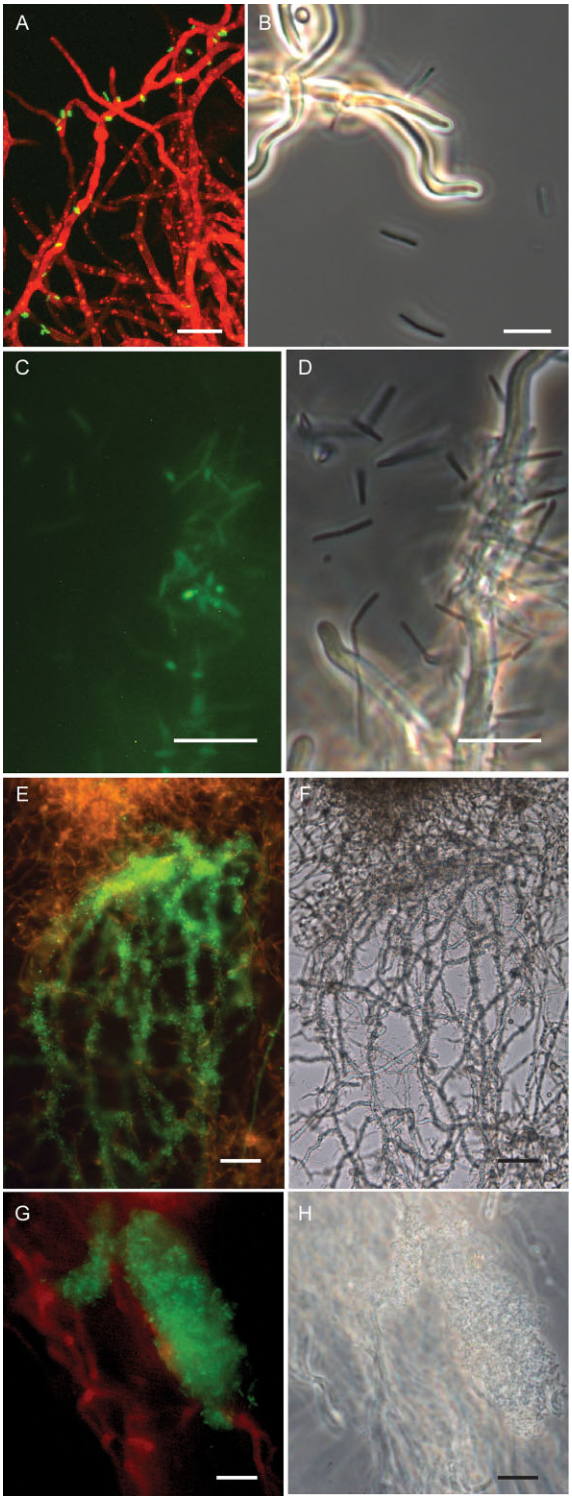

Fig. 2. Time-course of the *B. subtilis* attachment to the *A. niger* hyphae. Photographs (A) to (H) were taken 2 h, 4 h, 8 h and 24 h after co-inoculations respectively. The left panel in (A) is a confocal image of *B. subtilis* expressing green fluorescent protein and *A. niger* expressing red fluorescent protein, while panel (B) shows a bright field image. Left panels (C) to (H) show the confocal images of GFP-labelled *B. subtilis*, while the right panels show the corresponding bright field images. Scale bars indicate 10 μm.

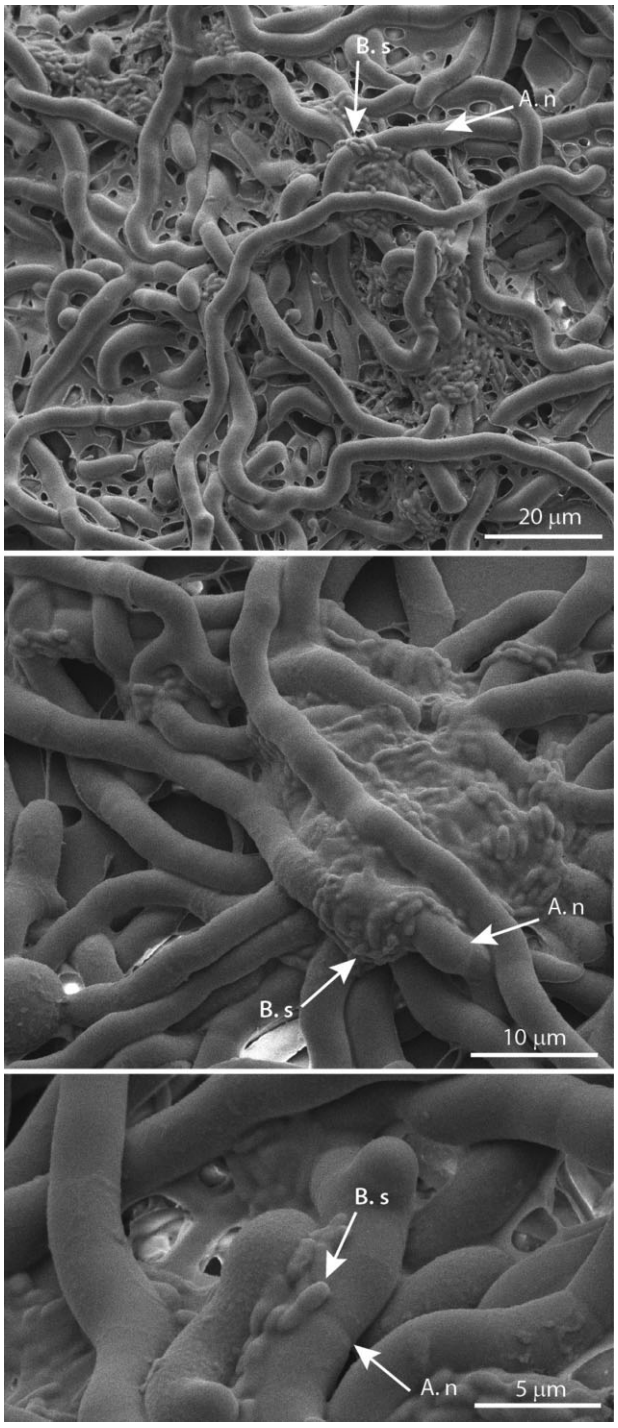

Fig. 3. Scanning electron microscopy images of the *B. subtilis* cells on the *A. niger* hyphae at 4 h after co-inoculation. The arrows point either *A. niger* hyphae (A.n) or *B. subtilis* (B.s).

was visualized using scanning electron microscopy (Fig. 3). Layers of *B. subtilis* cell were clearly visible on the washed *A. niger* mycelium suggesting a strong interaction between the bacterium and the fungi.

Aspergillus niger mycelium was incubated at 95°C for 10 min to kill the cells. When the bacterial cells were then added to the culture, *B. subtilis* showed no attachment; instead, the cells remained planktonic in the liquid phase of the medium (Fig. S3). In terms of stability of the co-culture, after 1 week, a thin layer of *B. subtilis* was observed at the interface between the air–liquid medium (i.e. pellicle formation) and *A. niger* colonies grew on top of this layer (Fig. 4).

Transcriptional response of A. niger *to the attachment of* B. subtilis

For the examination of the transcriptional response of *A. niger* to the presence of *B. subtilis* cells, *A. niger* samples were taken in the presence or absence of bacterial cells. Based on the time-course of the attachment of *B. subtilis* to *A. niger* hyphae, samples were taken after 4 h of co-incubation (see Experimental procedures) to be at the earliest stage of the interaction and to have enough bacterial material for a simultaneous study of both interacting partners. When bacterial cells were present, up to 5.4% (786 genes) of the *A. niger* genome showed a significantly altered expression. Among these genes, 53% were downregulated and 47% were upregulated (Table S1). Quantitative reverse transcription polymerase chain reaction of selected genes proved the validity of the transcriptome data (Table S2).

The differentially expressed genes were organized based on their function (Table S1) according to Functional Catalogue (FunCat; Ruepp *et al*., 2004), go-term Aspergillus Genome Database (Arnaud *et al*., 2010) and BioMet Toolbox analyses (Andersen *et al*., 2008). The two most represented groups included genes involved in detoxification/secondary metabolite production (15%) and genes related to carbon metabolism (10%); in both cases, more than two thirds of the genes were downregulated. Genes involved in transcription, fatty acids metabolism and protein fate account for 5–10% of the differentially regulated genes, while genes involved in protein synthesis, cell fate, amino acids metabolism and protein transport constituted around 4–5% of genes. Genes involved in fungal cell wall turnover, energy production, aromatic and nucleotide metabolism, mitochondrion, heat shock proteins and putative regulators were below 4%. Finally, 25% of the genes with altered expression are coding for hypothetical proteins with unknown function. Interestingly, various genes related to the fungal defence mechanisms such as antibiotic [cephalosporin C biosynthesis protein, several non-ribosomal protein synthesis (NRPS), polyketide synthesis (PKS)] or stress responses (epoxide hydrolases) were downregulated (Fig. 5).

While most of the genes involved in carbon metabolism are downregulated (68%), some genes of the

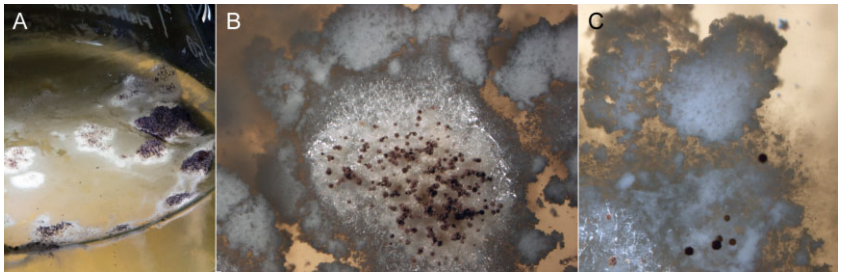

Fig. 4. Co-cultivation of *A. niger* and *B. subtilis* in liquid medium after 1week.
A. This photograph was taken with a Nikon digital camera D5100.
B and C. These photographs were taken with a Nikon stereomicroscope SMZ1000.

pentose catabolic pathway (PCP) and the pentose phosphate pathway were upregulated (95% confidence level): xylitol dehydrogenase (EC 1.1.1.9) that oxidizes the xylitol into D-xylulose, D-xylulose 5-phosphate/D-fructose 6-phosphate phosphoketolase (EC 4.1.2.9) that catalyzes the conversion of D-fructose 6-phosphate to D-xylulose 5-phosphate, and ribulose-5-phosphate-epimerase (EC 5.1.3.1) and transketolase (EC 2.2.1.1) both of which generate D-xylulose-5-phosphate. Moreover, a uridine diPhosphate-glucose 4-epimerase (EC 5.1.3.2) involved in the Leloir pathway and a mannose 6-phosphate isomerase (EC 5.3.1.8) both converging to D-fructose 6-phosphate were also upregulated in the presence of *B. subtilis* together with two alcohol dehydrogenases [nicotinamide adenine dinucleotide phosphate (NADPH/NADP+)].

Half of the genes involved in cell wall biosynthesis and remodelling were upregulated, including genes that are responsible for chitin synthesis and others that code for surface proteins. Notably, the gene coding for FluG, was highly upregulated. In *A. nidulans*, FluG is involved in conidiophore formation and germination (Lee and Adams, 1994; Breakspear and Momany, 2007); however, the exact molecular function of FluG in *A. niger* remains unclear. Ninety-seven and 70% of the *A. niger* differentially expressed genes involved in protein and nucleotide synthesis respectively, showed higher expression when interacting with *B. subtilis* than alone. Among the previously studied genes related to protein fate, 39% of the differentially expressed genes are proteases and these proteases are downregulated in the presence of *B. subtilis*. Most of the differentially expressed genes

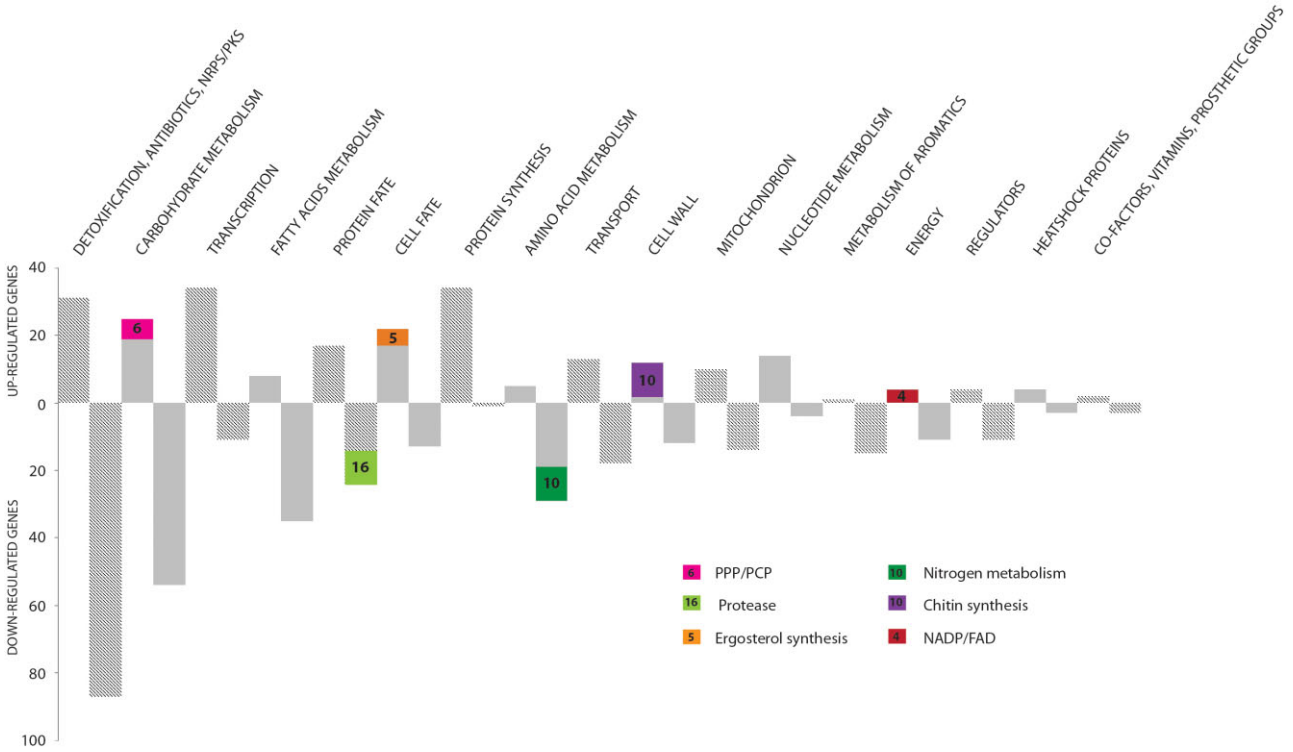

Fig. 5. Groups of genes differently expressed in *A. niger* in the presence versus absence of *B. subtilis* 4 h after co-inoculation. The vertical axis represents the number of up- and downregulated genes. Particular groups of interest are highlighted with colours and the number of genes involved.

involved in amino acids metabolism (85%) were downregulated as well as genes involved in nitrogen metabolism, such as the AmdS-regulon and the gene coding for the nitrate assimilation regulatory protein NirA (Daboussi *et al*., 1991). In *A. nidulans*, the enzymes involved in nitrate assimilation are under the control of two positive regulators, NirA and AreA (Narendja *et al*., 2002). NirA and AreA act synergistically to mediate nitrate/nitrite induction and nitrogen metabolite derepression respectively (Schinko *et al*., 2013). In this study, the expression of *areA* was not affected by the presence of *B. subtilis*. The expression of *nirA* and *areA* genes were confirmed by reverse transcription quantitative polymerase chain reaction (RT-qPCR) experiments (Table S2). To assay the growth differences suggested by the altered expression of metabolic genes, ergosterol levels in *A. niger* were determined in the presence and absence of *B. subtilis*. Slightly more ergosterol was measured when *A. niger* was grown in the presence of *B. subtilis*, although the differences were not significant (paired *t*-test, $P = 0.12$, $n = 6$; Table S3).

Genes involved in detoxification and genes coding NRPS and PKS are the second biggest group of genes differentially expressed between the *B. subtilis*-coated *A. niger* and free *A. niger*. Seventy-four per cent of these genes are downregulated during the BFI. Filamentous fungi contain a number of metabolic pathway clusters that are also necessary for the biosynthesis of primary or secondary metabolites (Keller and Hohn, 1997). Secondary metabolite gene clusters are relatively small in size, often containing less than 15 genes arranged adjacent to one another on the chromosome. The exact functions of these clusters are not all described yet. In this study, a cluster comprising of six genes including a putative synthase, two hydroxylases, transporter and regulator was identified. This gene cluster is downregulated in the presence of *B. subtilis*. Moreover, this cluster of genes is co-upregulated on different substrates (Fig. S4). The predicted function given for the key 'backbone' enzyme by the Secondary Metabolite Unique Region finder (SMURF) (Khaldi *et al*., 2010) is a demethylallyl tryptophan synthase (DMATS), which is involved in ergot alkaloid biosynthesis. Ergot alkaloids are toxins and important pharmaceuticals (Metzger *et al*., 2009). Interestingly, another predicted function for one of the synthases of this cluster was found as a NRPS (Inglis *et al*., 2013). This type of cross-chemistry has been described in *A. nidulans* (Andersen *et al*., 2013). However, further experiments are required to prove the presence of secondary metabolites connected to this gene cluster. From the 26% of the genes with increased expression in this functional category, no genes coding for antibiotics or clearly targeting *B. subtilis* presence were identified.

Transcriptome profiling of B. subtilis *attached to the mycelia of* A. niger

When *B. subtilis* and *A. niger* are grown together, part of the *B. subtilis* population attaches to the mycelia of *A. niger* while the other part of cells remains in the medium fraction. To identify the first transcriptional responses of *B. subtilis* during this direct interaction with *A. niger*, the two *B. subtilis* populations were separated. *Bacillus subtilis* that attaches to the mycelia was filtered from the medium (see Experimental procedures) and therefore separated from the cells in the planktonic phase. Mild RNA extraction from the mixed samples facilitated the enrichment of bacterial RNA over the fungal RNA (Fig. S5). The transcriptome of the two populations of *B. subtilis* was compared. In total, the expression of 279 genes was significantly changed ($\log FC > 1$ or < -1, adjusted *P* value $\leq 1.0 \times 10^{-3}$), around 7% of the genes on the *B. subtilis* genome (Table S4). The differential expression of selected genes was validated by RT-qPCR experiments (Table S5). The functional categories of genes with significant up- and downregulated transcription are presented in Fig. 6. The genes that have increased expression in the mycelia attached fraction of *B. subtilis* belong to late sporulation (spore coat) and germination genes and are related to anaerobic metabolism. Interestingly, early sporulation genes (e.g. *spoII* and *spoIII* genes) were not significantly altered. The late sporulation and germination genes identified in our comparison belong to the GerE, GerR and SpoVT regulons. These regulators increase the expression of late sporulation genes directly and indirectly (Eichenberger *et al*., 2004; Cangiano *et al*., 2010; Ramirez-Peralta *et al*., 2012), and therefore affect the germination protein level. Luria–Bertani medium does not support high sporulation of *B. subtilis* (i.e. in the absence of *A. niger*) (Vishnoi *et al*., 2013). However, spores of *B. subtilis* were observed on the hyphae after prolonged incubation that might explain the higher transcript level of spore coat-specific genes in the attached population of cells (Fig. S6). No significant difference in spore content could be detected in the 2 days of grown *B. subtilis* cultures in the presence or absence of *A. niger* ($1.2 \pm 0.53\%$ and $1.69 \pm 0.98\%$ sporulation efficiency of *B. subtilis* grown in the presence and absence of *A. niger* respectively). In cells that interact with the mycelium, the *narGHIJ*, *narK-fnr*, *arfM* genes that are regulated by the transcription factor FNR were upregulated. The *nar* operon is coding for enzymes involved in nitrate respiration in *B. subtilis* (Nakano *et al*., 1997). Also other genes related to anaerobic metabolism, *ldh-lctP*, *hemN* and *cydABCD* genes, were enhanced in the interacting cells. These results might indicate a decreased oxygen level next to the interacting cells and changes of *B. subtilis* metabolism from aerobic respiration to anaerobic

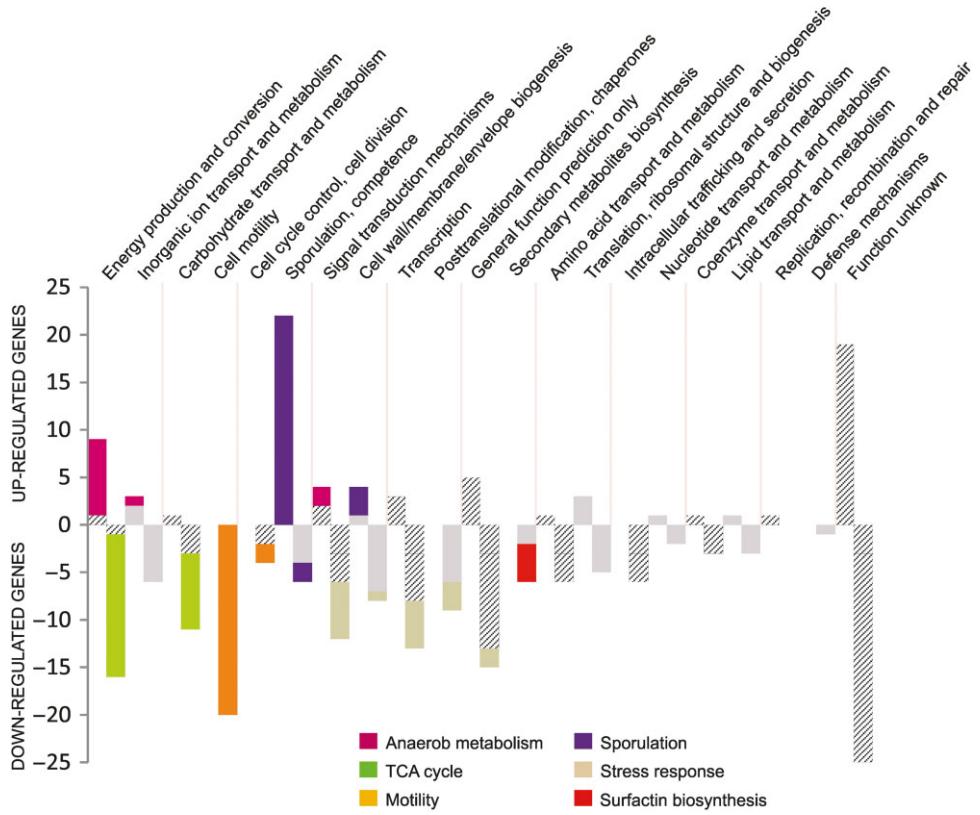

Fig. 6. Groups of genes differently expressed in *B. subtilis* in the cells attached versus non-attached to the fungal hyphae 4 h after co-inoculation. The vertical axis represents the number of up- and downregulated genes. Particular groups of interest are highlighted with colours.

fermentation. In line with this, genes coding for enzymes of the TCA cycle and aerobic respiration are downregulated in cells attached to the mycelium. Additionally, the expression of motility and autolysis genes was also reduced, suggesting a shift from planktonic to sessile growth style. Indeed, we observed motile cells in the supernatant of the medium that attaches to the mycelium and attenuates motility (see Supplementary Movie S1). Furthermore, we observed decreased expression of various stress response-related genes in *B. subtilis* in the attached cells. In particular, genes that belong to the CtsR and SigW regulons were downregulated. CtsR and SigW modulate the expression of genes related to protein control and cell wall stress/antibiotic production respectively (Derré *et al*., 1999; Helmann, 2006). Also, members of the YwrH regulon that are related to cell surface maintenance were downregulated. Finally, expression of the *srfA* operon was downregulated (−1.04- to −1.36-fold) in cells that are attached to the hyphae (Table S4). The *srfA* operon encodes a non-ribosomal surfactin synthetase complex (Nakano *et al*., 1991). Surfactin is not only a powerful biosurfactant (Grangemard *et al*., 2001) and a paracrine-signalling molecule (López *et al*., 2009b), but also functions as an antibiotic (Singh and Cameotra, 2004). Surfactin produced by *B. subtilis* isolates was previously reported to also inhibit various *Aspergillus* species (Mohammadipour *et al*., 2009; Velho *et al*., 2011). Analysis of the secreted metabolite profiles of the *B. subtilis*–*A.niger* co-culture and *B. subtilis* mono-culture showed that the amount of surfactin was lower in the presence of *A. niger* compared with the pure *B. subtilis* culture (Fig. 7). No significant difference could be identified in the bacterial cell counts of the cultures used ($1.04 \pm 0.07 \cdot 10^9$ and $1.27 \pm 0.24 \cdot 10^9$ colony-forming unit·(cfu) ml^{-1} in the presence and absence of *A. niger* respectively). To further verify that this was indeed surfactin, a *srfAA* mutant strain of *B. subtilis*, which is known to lack surfactin production (López *et al*., 2009a), was analyzed and compared with the 3610 strain. The peak corresponding to surfactin was absent in the supernatant of mutant strain (Fig. S7). In summary, our transcriptome analysis suggests that in addition to metabolism, surfactin production is altered in *B. subtilis*.

Discussion

When *A. niger* is grown on agar plates together with vegetative cells of the soil bacterium *B. subtilis*, the two

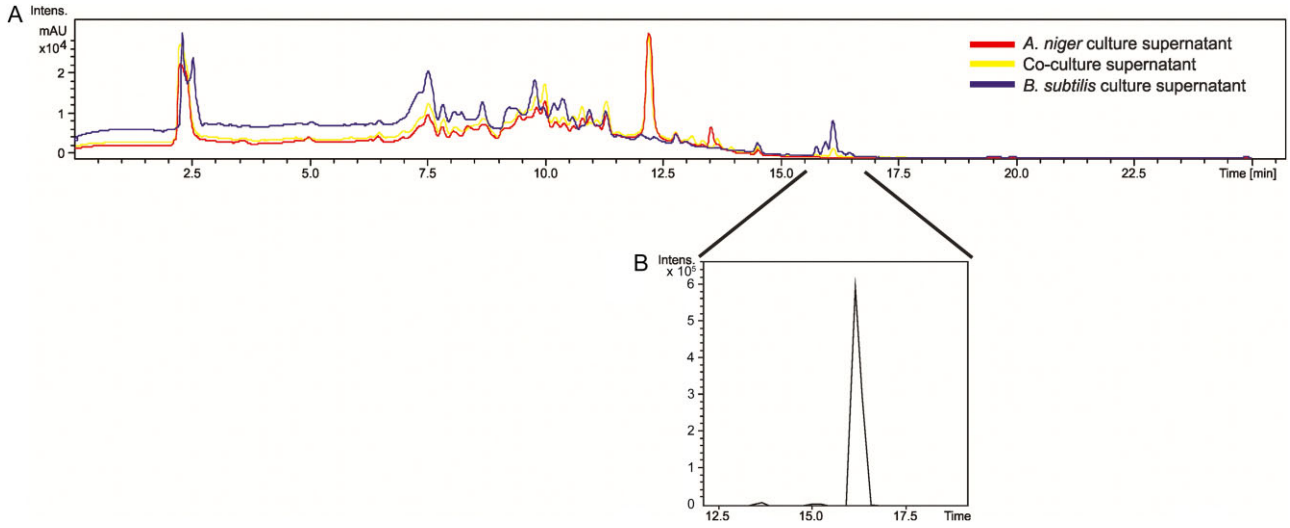

Fig. 7. High-performance liquid chromatography and liquid chromatography–mass spectrometry analysis of media supernatants.
A. HPLC-UV chromatogram profiles of the culture supernatants shown for *A. niger* alone (red), *B. subtilis* 3610 coincubated with *A. niger* (yellow) and *B. subtilis* 3610 alone (blue).
B. Mass spectrometric analysis, extracted ion chromatogram (EIC) m/z 1034.8 $[M–H]^-$ profile for surfactin.

microorganisms interact. The agar penetrating mycelium of *A. niger* is covered with *B. subtilis* cells while the aerial hyphae were not colonized by the bacteria. As observed both in solid medium and in liquid medium, *B. subtilis* attaches to and grows on particular parts of the *A. niger* hyphae, but not all hyphae are equally colonized by the bacterial cells. The specificity of the observed BFI was controlled in different experiments. While the undomesticated *B. subtilis* 3610 strain attached to the mycelia, the domesticated 168 strain did not show interaction. Also, heat treatment of the fungal mycelium prevented the interaction of *B. subtilis* with *A. niger*. While this kills the fungal cells, it does not change the chitin in the fungal cell wall, suggesting that the examined BFI is not due to chitin attachment as previously described for the co-biofilm between *Salmonella enterica* and *A. niger* (Brandl *et al*., 2011), and it is not activated by a heat stable compound or protein. Whether this means that the attachment is dependent on living *A. niger* mycelium or on specific cell wall components affected by the heat treatment is not clear at this point. During the attachment of *B. subtilis* cells to *A. niger*, a difference among various parts of the hypha can be observed, the hypha are targeted at specific regions while other parts are not covered. Although *Paenibacillus polymyxa* polar attachment to *Fusarium oxysporium* hyphae has been shown, no preference for hyphal tips, branch initials or phialides was observed in our experiments (Dijksterhuis *et al*., 1999). Hyphal differentiation has been described for the exploring hyphae of *A. niger* where the protein production showed alteration among distinct hyphae, including high and low protein producer parts (Vinck *et al*., 2005). Moreover, *B. subtilis* also attaches to the mycelia that were washed several times with phosphate buffer before the addition of the bacteria, suggesting that cell wall bound molecules or anchored and not soluble small peptides might play a role in the observed BFI with *B. subtilis*. The attachment of *B. subtilis* to *A. niger* hyphae is notably fast as within 4 h in shaken conditions, clusters of non-motile bacteria were observed. The co-culture was stable in a longer time frame as both microorganisms were able to grow together after a week of co-cultivation.

Transcriptional or proteomic characterizations of BFIs generally concern only either the bacteria or the fungi (Schrey *et al*., 2005; Deveau *et al*., 2007; Maligoy *et al*., 2008; Schroeckh *et al*., 2009; Holcombe *et al*., 2010; Moretti *et al*., 2010). Previous studies on dual transcriptome of BFI were restricted to indirect interaction of bacteria and fungi via chemical communication (Mela *et al*., 2011). Separating the bacterial cells that interact with the fungal hyphae from those that stay in the medium helped us to determine the transcriptional differences between cells in the same culture that commit to distinct phenotypic traits (i.e. attachment to hyphae versus planktonic growth).

During the examined BFI, the metabolism of *A. niger* is actively involved in nucleotide synthesis, transcription and protein synthesis. Among the genes involved in carbon metabolism, the pentose phosphate and PCPs are upregulated, leading to the production of NADPH. Furthermore, genes involved in NADPH conversion are also upregulated. NADPH functions as a reductant in various anabolic pathways, such as nucleic acid synthesis. Moreover, genes involved in chitin and ergosterol

synthesis were upregulated and slightly more ergosterol has been measured when *A. niger* was grown in the presence of *B. subtilis*, although the differences were not significant (Table S3). Chitin and ergosterol are the two main components in fungal cell walls and are used as markers for living fungal biomass determination (Ekblad *et al.*, 1998). These data suggest that *A. niger* growth might be slightly enhanced, but not repressed by the presence of *B. subtilis.*

Similar to the fungal host, microarray analysis showed altered expression of *B. subtilis* metabolic genes. The shift from aerobic to anaerobic metabolism might be explained by the attachment of bacterial cells to the hyphae and/or growth of the bacteria in a compact community (biofilm). The fungal cells might consume the oxygen locally resulting in even lower oxygen availability. In contrast, when *B. subtilis* cells were located in the supernatant of the aerated culture, sufficient oxygen was available. When the bacterial cells colonized the hyphae, motility was reduced as expected during attachment. Similarly, genes related to bacterial stress response are downregulated during the attachment. These transcriptional changes suggest that *B. subtilis* interaction with *A. niger* is not only a surface attachment, but colonization of the hyphae is an active process in which the bacterium rewires metabolism, differentiation (i.e. motile to sessile form), antimicrobial production and general stress response. A similar transition from motile to sessile form is observed during biofilm formation of *B. subtilis* (Vlamakis *et al.*, 2013). Microarray analysis of *B. subtilis* biofilm formation has so far mainly focused on various mutants or use of an air–liquid interface biofilms (so called pellicle) (Ren *et al.*, 2004). However, during pellicle formation, anaerobic genes are repressed compared with the cells in the medium fraction in contrast to our results. Also, the expression of genes related to biofilm formation of *B. subtilis* was not significantly altered in our experimental setup.

The majority of previous molecular biology studies on BFI revealed how one microorganism affects the growth and development of the other microorganism by producing various metabolites or small peptides that possess microbe-inhibiting activity. In specific cases, such a BFI can awake the production of secondary metabolites. Soil-dwelling actinomycetes induce the production of secondary metabolites in *A. nidulans* that are not produced under laboratory conditions (Schroeckh *et al.*, 2009; Nützmann *et al.*, 2011; Scherlach *et al.*, 2013). In contrast, our transcriptome profiling of *A. niger* and *B. subtilis* suggests that the production of antimicrobial compounds was not increased, but rather reduced at least in *B. subtilis*. Mutual interactions are not unprecedented among microbes [e.g. *Lactobacillus–Streptococcus* interaction (Sieuwerts *et al.*, 2010)], but mutual interactions of bacteria and fungi are less described. It will be interesting to examine the secreted protein profiles of *A. niger* and *B. subtilis* during the BFI under enzyme-producing conditions (e.g. agricultural by-products induce the plant cell wall-degrading enzymes production by *A. niger*) and examine how the interaction alters the biotechnological potential of both organisms (e.g. enzyme production properties, growth or secondary metabolites). Also, our experiments suggest that in the environment, these two microorganisms might affect each other without producing inhibitory molecules. The respective number of fungal and bacterial cells depending on the environmental pressure may play an important role in the equilibrium between the two microorganisms. The co-occurrence and attachment of *B. cereus* on arbuscular mycorrhizal hyphae was previously reported presenting an environmentally relevant example of a *Bacillus*–fungi interaction (Arturson and Jansson, 2003). Our report presents a laboratory example of a *Bacillus–Aspergillus* interaction that justifies further investigation under environmental conditions.

Experimental procedures

Strains and culture conditions

Bacterial cultures. Bacillus subtilis NCIB3610 (3610) (Branda *et al.*, 2001), DS908 [3610 harbouring a P*hag-gfp* construct (Kearns and Losick, 2005)], DL107 [3610 *srfAA*::MLS (López *et al.*, 2009a)] strains were cultivated in rich TY medium (1% Bacto tryptone, 0.5% Bacto yeast, 0.5% NaCl and 0.1 mM of $MnCl_2$) at 30°C and 15 g l^{-1} agar was added in case of solid medium. For each co-cultivation, *B. subtilis* inoculum was picked from plates with newly grown culture. The final concentration of bacteria in the co-cultures was 5 10^6 cfu ml^{-1}.

Fungal cultures. Aspergillus niger CB-A119.1 and FP102 [harbouring P*gpdA-gfp* and P*gpdA-dtomato* constructs respectively (de Bekker *et al.*, 2011, and this study)] were inoculated at 10^4 spores ml^{-1} and cultivated in TY medium at 30°C, 250 r.p.m. for 15 h.

Co-cultivations on plates. Bacillus subtilis of 0.2 µl ($1.5{\cdot}10^9$ cfu ml^{-1}) added to 2 µl of *A. niger* ($2{\cdot}10^6$ spores ml^{-1}) was inoculated at 30°C in the centre of TY agar plates and incubated for 8 days.

Co-cultivation in liquid culture. Aspergillus niger was pre-cultured overnight (15 h) in liquid TY medium, and *B. subtilis* was subsequently added to the pre-cultures at a final concentration of $5{\cdot}10^6$ cfu ml^{-1}. The shaken co-cultivations were incubated at 30°C, 150 r.p.m. for 2–40 h before samples were taken for analysis. Samples for microarray analysis were taken at 4 h.

Fungal RNA isolation, quality control, microarray analysis and RT-qPCR experiments

Mycelium from three *A. niger* cultures in absence and in presence of *B. subtilis* was pooled on Miracloth and

immediately frozen in liquid nitrogen. Total RNA for microarray analyses was isolated from frozen-grinded mycelium by Trizol extraction according to the manufacturer's instructions. RNA quantity and quality were determined on a Nanodrop spectrophotometer, and integrity was tested on an Agilent 2100 Bioanalyser. The co-cultures and control experiments were done in triplicate resulting in six samples (three free *A. niger* and three *A. niger* in presence of *B. subtilis*).

dsmM_ANIGERa_coll511030F GeneChip® containing 14 554 probesets was used to measure gene expressions in *A.niger*. Fragmented and labelled DNA was hybridized to the chips, washed, stained and scanned. The dsmM_ANIGERa_coll511030F library and platform information is deposited at Gene Expression Omnibus under number GPL6758. Probe synthesis and fragmentation were performed at ServiceXS (Leiden, The Netherlands) according to the GeneChip Expression Analysis Technical Manual (Affymetrix 2002. GeneChip Expression Analysis Technical Manual. Affymetrix, Santa Clara, CA). DSM (Delft, The Netherlands) proprietary *A. niger* gene chips were hybridized, washed, stained and scanned as described in the GeneChip Expression Analysis Technical Manual. Microarray data was analyzed using the Bioconductor Affy tool package (http://www.bioconductor.org) and CyberT (Kayala and Baldi, 2012) with parameters described as in Gruben and colleagues (2012). Gene expression variation with P value < 0.05 and fold change $\leqq 1.5$ were considered significant (Table S1). Secondary metabolite biosynthesis genes were predicted by SMURF (Khaldi *et al.*, 2010) and antiSMASH (Medema *et al.*, 2011). Protein functional categories were classified by FunCat (Ruepp *et al.*, 2004). Putative regulators were predicted by the Pfam and Superfamily domains described by Shelest (2008). Microarray data have been deposited in the Gene Expression Omnibus database (Accession No. GSE46187). Quantitative PCR experiment for *A. niger* has been performed as following. Primer pairs were designed using the software Primer express 3.0 (Applied Biosystems, Warrington, UK). The primers were tested to determine the optimal primers concentrations and efficiency. All primers had between 98% and 107% efficiency. The sequences of the primers and optimal primer concentration are listed in Table S7A. A total of 4 μg of RNA was treated with DNase using a NucleoSpin RNA II kit (Macherey-Nagel, Germany) and 2.5 μg of RNA converted into cDNA (ThermoScript™ RT–PCR system, Invitrogen, Carlsbad, USA) according to the instructions of the manufacturer. The amount of target cDNA was normalized to the level of *gpd* cDNA. To confirm the absence of genomic DNA contamination, a reaction without the reverse transcriptase was performed for each sample. cDNA of 25 ng was assayed in triplicate in a final volume of 20 μl of qPCR reaction containing optimized concentration of each primer and 10 μl of ABI Fast SYBR Master Mix (Applied Biosystems, Foster City, USA). Reactions were carried out in a ABI 7500 fast real-time PCR system (Applied Biosystems, Foster City, USA), with the following cycling conditions: 95°C 20 s, followed by 40 cycles of 95°C 3 s and 60°C 30 s. The dissociation curve was generated to verify that a single product was amplified. Standard curves for each gene were prepared using genomic DNA.

Bacterial RNA isolation, transcriptome and RT-qPCR experiments

Bacillus subtilis was grown in the presence of *A. niger* for 4 h as described above. The mycelium and the attached bacteria were separated from the non-attached *B. subtilis* cells via filtration through Miracloth. The mycelia and attached bacteria were briefly rinsed with TY medium, dried and the sample was frozen in liquid nitrogen. Bacterial cells in the flow-through medium fraction were harvested by centrifugation at 10 397*g* for 1 min. RNA was isolated by resuspending the cell pellet in 400 μl of TE (10 mM of Tris·HCl, 1 mM of EDTA, pH 8.0). Subsequently, 400 μl of lysozyme solution (15 mg ml^{-1}) and 5 μl of RiboLock (Thermo Scientific) was added followed by incubation at 37°C for 30 min. The RNA extraction was performed with the Macaloid/Roche method as described before (van Hijum *et al.*, 2005; Kovács and Kuipers, 2011) with the following modifications. The bead beater treatment was omitted to reduce lysis of fungal cells and RiboLock was supplied. The replacement of bead beating with the lysozyme treatment as described above facilitates the enrichment of bacterial RNA versus fungal RNA (Fig. S5). The concentration of the extracted RNA was measured using NanoDrop (Thermo Scientific) and quality was analyzed using an Agilent 2100 Bioanalyzer (Fig. S5). RNA of 5 μg was used for cDNA synthesis as described earlier (Kovács and Kuipers, 2011). Dylight-635 and Dylight-535 of 0.6 μg each (Thermo Scientific) labelled cDNA was mixed and hybridized for 17 h at 60°C to 8 × 15 K Agilent slide according to the general protocol provided by the manufacturer. The microarray slides contain specific oligonucleotides for 4171 open reading frames of *B. subtilis* 168 and were prepared according at Agilent Technologies. Slides were washed for 10 min in wash solution I (6 × SSC with 0.005% Triton X-102) and for 5 min in wash solution II (0.1 × SSC with 0.005% Triton X-102) precooled to 4°C. Slides were air dried and scanned in GenePix 4200AL (Axon Instruments, CA, USA) microarray scanner. Fluorescent signals were quantified using GENEPIX PRO software and further processed and normalized using standard routines included in the Limm aR package (Linear Models for Microarrays Data) (Smyth 2004). Genes with an adjusted P value of lower than 10^{-3} (logFC > 1 or < -1) were considered significantly affected (Table S4). To assess cross hybridization of *A. niger*-related cDNA on the microarray slides, labelled *A. niger* cDNA was hybridized against *B. subtilis* cDNA, including a dye swap. Spots resulting in high signal from the *A. niger* cDNA was omitted from the analysis (Table S6). Microarray data has been deposited in the Gene Expression Omnibus database (Accession No. GSE46682).

qPCR experiments for *B. subtilis* have been performed as described before (Mironczuk *et al.*, 2011), except that cDNA from the RNA samples was obtained identically to the microarray experiments above. Briefly, RNA samples obtained as described above for the microarray experiments were treated with RNase-free DNase I (Thermo Fisher Scientific, St. Leon-Rot, Germany) for 60 min at 37°C in DNaseI buffer (10 mM of Tris • HCl (pH 7.5), 2.5 mM of $MgCl_2$, 0.1 mM of $CaCl_2$). Samples were purified with the Roche RNA isolation Kit. Quantification of cDNA was performed on an iQ5 Real-Time PCR System (BioRad, Hercules, CA) using Bio-Rad iQ supermix. The primers used are listed in

Table S7B. Reverse transcriptions were performed in duplo on each RNA samples, and PCR reactions were performed in triplo for each cDNA sample. The amount of target cDNA was normalized to the level of *girB* cDNA using the RELATIVE EXPRESSION SOFTWARE TOOL (Pfaffl *et al.*, 2002).

The correlation between the microarray data and the RT-qPCR experiments is presented in Tables S2, S5 and Fig. S8.

Alkaline ergosterol extraction

Twelve independent cultures were inoculated in 20 ml of TY medium with 10^4 spores ml^{-1} of *A. niger* and incubated at 30°C, 250 r.p.m. for 15 h. *Bacillus subtilis* was subsequently added to six of the pre-cultures at a final concentration of $5{\cdot}10^6$ cfu ml^{-1}. The six mono-cultures of *A. niger* and the six co-cultures of *A. niger* and *B. subtilis* were incubated at 30°C, 150 r.p.m. for 6 h. The 20 ml of three Erlenmeyers were pooled; the mycelium was harvested through a Miracloth filter and rinsed with ACES buffer. The pellet was stored at –20°C. This protocol was repeated three times independently, which makes the final number of samples to 12 (6 mono-cultures and 6 co-cultures). The alkaline ergosterol extraction was performed the following day as described by van Leeuwen and colleagues (2008).

Microscopy

Green fluorescent protein (GFP) and dTomato fluorescence was monitored with an Axioskop 2 plus microscope (Zeiss, Germany) equipped with a HBO 100 W mercury lamp and a Photometrics Cool SNAP camera (1392 × 1024 pixels) using standard FITC and tetramethylrhodamine filters respectively. Confocal laser scanning microscopy was performed using an inverted Zeiss LSM5 system equipped with a PLAN-Neofluar 20×/0.50 objective lens. The GFP protein was excited with the 488 nm laser line and fluorescence was detected at 500–550 nm bandpass. The dTomato protein was excited with the 543 nm laser line and fluorescence was detected with a long pass LP560 emission filter. Bright field images were made using the transmission channel. Laser intensity was kept to a minimum to reduce photobleaching and phototoxic effects. Images were captured as *z*-series of optical sections. The data sets were displayed as maximum intensity projections (1024 × 1024 pixels) using ZEISS software.

Co-cultivated bacteria and fungi (4 h) were used for cryoSEM. Fungal pellets were brought on a polycarbonate filter (0.5 μm, Profiltra, Almere, The Netherlands) on a moisted Whatman paper filter round. Excess fluid was taken up through the polycarbonate filter by pressing it gently against the paper filter with a tweezer. Then a small square part of the filter was excised with a surgical blade and quickly placed in a small copper cup for electron microscopy. The piece of filter was glued to the copper cup with Tissue Tek (KP-Cryoblock, Klinipath, Duiven, The Netherlands). This method allows the submersed fungal hyphae to be very well visible after freezing. A Petri-dish lid was put on the copper cup to keep the sample moist. The sample was snap-frozen in nitrogen slush. Samples were examined in a JEOL 5600LV scanning electron microscope (JEOL, Tokyo, Japan) equipped with an Oxford CT1500 Cryostation for cryo-electron microscopy (cryoSEM). Electron micrographs were acquired after sputter-coating by means of a gold target for three times during 30 s. Micrographs were taken at 5 kV in (PHOTO mode) and processed in Adobe Photoshop for size modifications.

High-performance liquid chromatography analysis of culture supernatant

The supernatants of the fungal and bacterial cultures were extracted with equal volumes of ethyl acetate, dried with sodium sulfate and concentrated to dryness with a rotary evaporator. The extracts were then re-dissolved in 1 ml of methanol and filtered with a 0.2 μm of polytetrafluoroethylene filter (Carl-Roth, Karlsruhe, Germany). 20 μl were then injected onto an Agilent 1100 series LC/MSD Trap LC-MS system with an electrospray ion source using an Agilent XDB-C8 5 μm, 4.6 × 150 mm column. The gradient was of methanol/0.1% (v/v) formic acid (H_2O) 10/90 in 15 min to 90/10, then 90/10 to 100/0 in 0.5 min and 100% methanol for 5.5 min with a flow rate of 1 ml min^{-1}.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Fig. S1. Co-culture of *B. subtilis* 168 with *A. niger* in shaken liquid culture.

Fig. S2. Co-culture of *B. subtilis* 3610 with *A. oryzae* in shaken liquid culture.

Fig. S3. Co-cultivation of *B. subtilis* 3610 with heat-treated hyphae of *A. niger* in shaken liquid culture.

Fig. S4. Identification of a gene cluster based on gene syntheny. The up (+) and downregulation (–) of indicated genes on various substrates show co-regulation of the gene cluster. Microarrays of *A. niger* grown on different substrates were performed using the same experimental protocol as described in this study. This figure demonstrates that the transcription of these genes is upregulated in *A. niger* when grown on various different raw plant biomass while it is downregulated in the presence of *B. subtilis* (see main text).

Fig. S5. Examination of the extracted RNA using Agilent Bioanalyzer. Gel-like format (A) and the electropherogram (B) of the samples are shown. Marker ladder (L), RNA extracted from the *B. subtilis* only sample (1), mixed *B. subtilis* and *A. niger* sample (2), and only *A. niger* (3) are shown. The prokaryotic (16S and 23S) and eukaryotic (18S and 28S) ribosomal RNA peaks are indicated above the electropherogram.

Fig. S6. Bright field image of *B. subtilis* spores attached on the hypha of *A. niger*. Scale bar indicates 10 μm.

Fig. S7. HPLC-UV analysis at 210 nm showing surfactin in the *B. subtillis* 3610 strain (grey) and the absence in the *B. subtillis srfAA* mutant (purple).

Fig. S8. Comparison of microarray and RT-qPCR results of validated genes for *A. niger* (A) and *B. subtilis* (B). The values obtained in the RT-qPCR experiment are presented on the *x*-axis, while the microarray data on the *y*-axis.

Table S1. List of all differentially expressed *A. niger* genes in the presence versus in the absence of *B. subtilis*.

Table S2. RT-qPCR validation of the *A. niger* microarray experiments.

Table S3. Alkaline ergosterol measurement.

Table S4. List of significantly up or downregulated *B. subtilis* genes ($\log FC > 1$ or < -1, P value $< 10^{-3}$) in the

A. niger interacting population of cells compared with the non-attached population of cells.

Table S5. RT-qPCR validation of the *B. subtilis* microarray experiments.

Table S6. *Bacillus subtilis* genes omitted from the microarray analysis due to cross hybridization of *A. niger* cDNA on the Agilent microarray slides.

Table S7. Oligonucleotides used for the RT-qPCR validation designed for *A. niger* (A) and *B. subtilis* (B).

Movie S1. *Bacillus subtilis* attachment to *A. niger* hypha. The first caption shows a group of *B. subtilis* cells already attached to the fungal hyphae and a motile-free cell. The second caption shows polar attachment of the bacteria to the fungal hyphae. The third caption shows attached and non-motile bacterial cells.