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Dual transcriptional profiling of a bacterial/fungal confrontation: *Collimonas fungivorans* versus *Aspergillus niger*

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Interactions between bacteria and fungi cover a wide range of incentives, mechanisms and outcomes. The genus *Collimonas* consists of soil bacteria that are known for their antifungal activity and ability to grow at the expense of living fungi. In non-contact confrontation assays with the fungus *Aspergillus niger*, *Collimonas fungivorans* showed accumulation of biomass concomitant with inhibition of hyphal spread. Through microarray analysis of bacterial and fungal mRNA from the confrontation arena, we gained new insights into the mechanisms underlying the fungistatic effect and mycophagous phenotype of collimonads. *Collimonas* responded to the fungus by activating genes for the utilization of fungal-derived compounds and for production of a putative antifungal compound. In *A. niger*, differentially expressed genes included those involved in lipid and cell wall metabolism and cell defense, which correlated well with the hyphal deformations that were observed microscopically. Transcriptional profiles revealed distress in both partners: downregulation of ribosomal proteins and upregulation of mobile genetic elements in the bacteria and expression of endoplasmic reticulum stress and conidia-related genes in the fungus. Both partners experienced nitrogen shortage in each other's presence. Overall, our results indicate that the *Collimonas/Aspergillus* interaction is a complex interplay between trophism, antibiosis and competition for nutrients.

Introduction

Bacterial mycophagy (Leveau and Preston, 2008) is a trophic behavior by which bacteria actively obtain nutrients from living fungal hyphae. For bacteria belonging to the genus *Collimonas* (de Boer *et al.*, 2004), mycophagy was demonstrated in a gnotobiotic sand system, in which bacterial numbers were shown to increase after invasion of common soil fungi like *Mucor hiemalis* and *Chaetomium globosum* (de Boer *et al.*, 2001). Follow-up studies revealed that mycophagy also occurred under natural circumstances (Höppener-Ogawa *et al.*, 2009). The mycophagous phenotype of collimonads

was recently explained as an adaptation to life under conditions of limited nutrient availability (Leveau *et al.*, 2010).

Currently unclear is the relationship between the mycophagous behavior of collimonads and their demonstrated ability to arrest fungal growth. It is fair to assume that these two phenomena share underlying mechanisms (Leveau and Preston, 2008). The ectomycorrhizal fungus *Laccaria bicolor* (Deveau *et al.*, 2007), arbuscular mycorrhizal fungus *Glomus mosseae* (Pivato *et al.*, 2009) and various soil fungi (de Boer *et al.*, 1998) all showed reduced fungal growth when co-inoculated with *Collimonas fungivorans* strain Ter331. While the detriment to the fungus was well documented in each case, it was not assessed if and how the bacteria caused it or benefited from it. Their antagonistic activity likely involved the production of an antibiotic compound, but whether this provided the bacteria an advantage over the fungus in competition for limiting nutrients, or helped them in a mycophagous manner, for example by causing hyphal damage and release of

fungal content, is not clear. These scenarios are not mutually exclusive, and both may be explored by mycophagous bacteria under conditions of nutrient limitation in the presence of fungi.

In preliminary confrontations of *C. fungivorans* Ter331 with *Aspergillus niger* on nutrient-poor plates, it was observed that (1) growth of this fungus was severely inhibited by the bacterium and (2) the presence of the fungus resulted in the accumulation of bacterial biomass. These results suggest simultaneous expression of the mycophagy phenotype (that is conversion of fungal biomass into bacterial biomass) and the production of one or more antibiotic compounds. Best known for its role as a common food spoiler, a 'cell factory' in the fermentation industry, and an opportunistic human pathogen, *A. niger* had its complete genome sequenced recently (Pel *et al.*, 2007). In addition, an Affymetrix microarray for transcriptomic interrogation is available, as is a large body of literature on *Aspergillus* mutants and their phenotypes. Similarly, genomic resources are available for *C. fungivorans* strain Ter331, including an annotated genome (Leveau *et al.*, 2010), a plasposon mutant library (Leveau *et al.*, 2006) and a large insert genomic library (Leveau *et al.*, 2004). We exploited the availability of genomic resources for both partners and took a transcriptomic approach to listen in on the dialog that goes on during their interaction on nutrient-poor plates. Typically, such approaches have been very valuable by offering new insight into the complexity of prokaryotic-host cross-talk (Barnett *et al.*, 2004; Tailleux *et al.*, 2008), including bacterial/fungal interactions (Schrey *et al.*, 2005; van Rij *et al.*, 2005; Deveau *et al.*, 2007; Maligoy *et al.*, 2008; Barret *et al.*, 2009). However, in many of these studies only one partner was profiled, treating the other one as a black-box component of the biotic environment. In the approach taken here, we profiled the transcriptomes of both the bacterium and the fungus, which allowed us to interpret any changes in gene expression in one organism in terms of the other's possible cause or effect.

Materials and methods

Strain cultivation and confrontation assay
C. fungivorans Ter331 (de Boer *et al.*, 2004) was routinely maintained on 1/10 TSB agar plates (composition of all media used in this study are listed in Supplementary Information). Growth of Ter331 on oxalate and citrate was tested on MM minimal medium. In preparation for the confrontation assay, *C. fungivorans* Ter331 was inoculated from 1/10 TSB agar into liquid 1/10 TSB medium. After o/n growth at 25 °C, bacterial cells were harvested by centrifugation, washed once with wash solution (0.25 g KH$_2$PO$_4$ per liter, pH 6.5), and resuspended to an optical density of 1 at 600 nm. Fungal strains used in this study include *A. niger*

strain N400 (CBS120.49), as well as its derivative 752.10, which is a non-oxalate producer (Ruijter *et al.*, 1999), and *Aspergillus westerdijkiae*, which was kindly provided by Robert Samson, CBS-KNAW, Fungal Biodiversity Centre, Utrecht, The Netherlands. In preparation for the confrontation assay, *Aspergillus* was grown on potato dextrose agar medium for 4 days at 30 °C. Conidia were harvested by washing with sterile saline solution (van der Veen *et al.*, 2009) supplemented with 0.005% Tween 80, filtered through Miracloth (Calbiochem, Nottingham, UK) to remove mycelial fragments, washed twice and resuspended in saline solution. The confrontation arena consisted of Petri dishes (9 cm diameter) containing 25 ml of WYA supplemented with 10 μg bromocresol purple per ml. Confrontations were set up in one of two ways, A and B. For setup A (shown in Figure 1), we placed in the center of a WYA plate an autoclaved 2 cm wide strip of Nuclepore Track-Etch polycarbonate membrane (Whatman, s'-Hertogenbosch, The Netherlands, catalog number 113506, 0.2 μm pore size), on top of which eight 2.5 μl droplets each containing 10^4 *A. niger* spores were equidistantly deposited. The polycarbonate membrane separated the mycelium from the agar medium, thus preventing the fungus from growing into the agar and allowing easy and complete removal of hyphal material for RNA extraction (see below). Next, eight 2.5 μl droplets of bacterial suspension were placed at a distance of 2.5 cm on either side of the membrane and streaked into a single line parallel to the edge of the membrane. Confrontation setup B (an example is shown in Figure 2a) was the same as A but did not feature a polycarbonate membrane. Also, *Aspergillus* was point inoculated, while *C. fungivorans* was inoculated as a single streak across the middle of the plate. For both setup A and B, plates were sealed with Parafilm and incubated at 20 °C. Control plates were inoculated as described above, but with only *Aspergillus* or *C. fungivorans* on the plate.
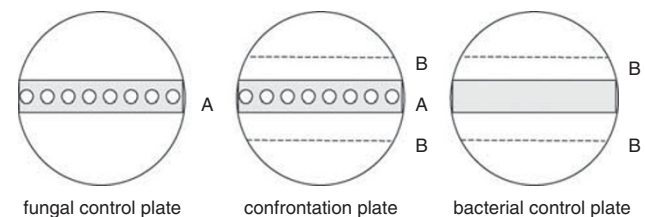

fungal control plate   confrontation plate   bacterial control plate

Figure 1 Experimental setup of the *Collimonas/Aspergillus* confrontation assay for RNA extraction. The setup features eight spot inoculations of *A. niger* (A) spores on a piece of polycarbonate membrane in the center of the plate, and two parallel lines of *C. fungivorans* inoculum (broken line B). Inclusion of the membrane was necessary for complete recovery of fungal mycelium from the agar surface for RNA extraction. The presence of the membrane did not affect the antagonistic impact of *Collimonas* on the fungus and production of slime by *Collimonas* as observed in the absence of the membrane (Figure 2). On the control plates, only one partner was inoculated, either *A. niger* (left panel) or *C. fungivorans* (right panel).
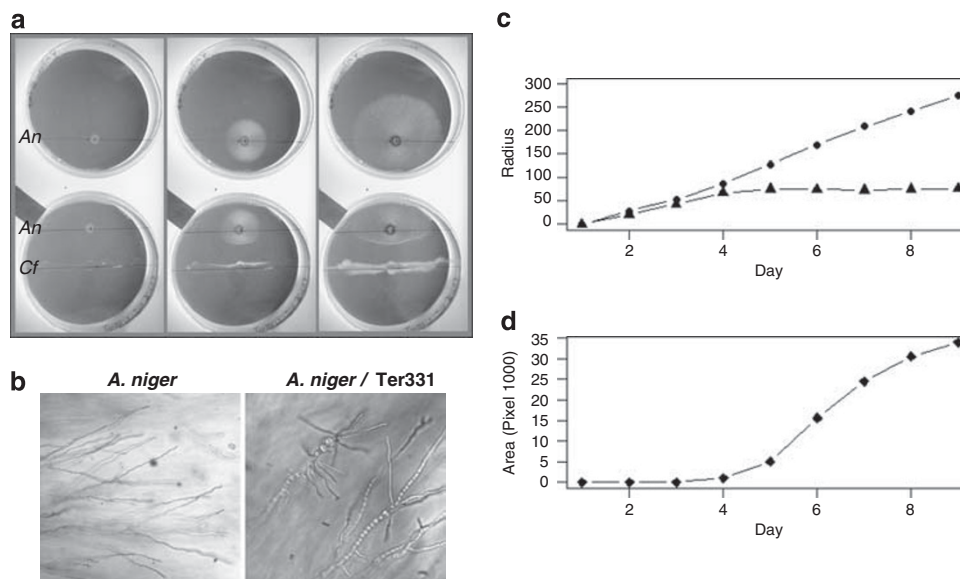
Figure 2 (**a**)Visualization of the *Collimonas/Aspergillus* confrontation, 2 (left), 4 (middle) and 7 (right) days after inoculation of *A. niger* (*An*) in the presence (bottom row) of *C. fungivorans* (*Cf*) or in its absence (top row). (**b**) Microscopic view of *A. niger* hyphae on the plate without (left) or with (right) *Collimonas*. (**c**) Radial hyphal growth (in pixels) of *A. niger* in the absence (circles) or presence (triangles) of *Collimonas*. (**d**) Production of slime by *Collimonas* in confrontation with *A. niger*. Not shown is the negative control: *Collimonas* in the absence of *A. niger* did not produce detectable levels of slime.

Assessment of oxalate concentrations, hyphal growth, slime production and agar acidification

All these were assessed using confrontation setup B. Oxalate concentrations were determined by extraction of agar from confrontation plates with 1 ml Milli-Q water per gram agar. The extract was filtered through a 0.25-μm filter. As a negative control, we used plates that were not inoculated with *Aspergillus*. As a positive control, we used agar that was supplemented with 300 μM oxalate. Extracts were tested for oxalate by high-performance liquid chromatography as described elsewhere (Ruijter *et al.*, 1999). For a standard curve, oxalate was used in the 0.1–10 mM concentration range. Hyphal growth of *Aspergillus* in the presence or absence of *Collimonas* was quantified by taking daily photographs and measuring the radius of the fungal colony (from the point of inoculation towards the center of the plate) using ImageJ (Abramoff *et al.*, 2004) and plotting these values (in pixels) as a function of time (Figure 2c). Slime production was quantified by ImageJ and expressed as the number of pixels in the area covering the plate in slime, plotted over time (Figure 2d). To test agar acidification and *Collimonas* slime production in response to different compounds, we streaked a horizontal line of Ter331 in the middle of a confrontation plate, incubated for 5 days at 20 °C, then using a sterilized cork borer punched a hole in the agar about 2 cm away from the Ter331 line and added 100 μl of the following stock solutions to the hole: (1) oxalate (100 mM), (2) oxalate (100 mM) adjusted with NaOH to pH 6.4, (3) citrate (100 mM), (4) citrate (100 mM) adjusted with NaOH to pH 6.5, (5) *N*-acetylglucosamine (400 mM), (6) glycerol (50%), (7) glucose (20%) or (8) water (control).

RNA isolation

Confrontation setup A was used to isolate RNA at two time points during the interaction between the bacterium and the fungus, that is 5 days after inoculation (time point T1), at which time the production of bacterial slime and slowdown of fungal growth were first apparent, and 1 day after T1 (T2). For fungal RNA isolation, ~60 mg mycelium was retrieved and pooled from seven confrontation plates. After grinding in a mortar with liquid nitrogen, total RNA was extracted using Trizol Reagent (Invitrogen, Breda, The Netherlands) following instructions of the manufacturer. Each RNA isolation was performed in duplicate for both treatments (that is the presence/absence of *C. fungivorans*) and both time points (that is T1/T2), resulting in eight fungal RNA samples. For bacterial RNA isolation, biomass was collected by retrieving and pooling cells from the same seven plates from which fungal RNA was isolated. After resuspension in pre-cooled (4 °C) wash solution and centrifugation at 3000 *g* at 4 °C, RNA was extracted from the bacterial cell pellet using Trizol Reagent. RNAprotect was not used, because it resulted in RNA of low quality. Each bacterial RNA isolation was done in duplicate for both treatments (presence/absence of *A. niger*) and both time points (T1/T2), resulting in eight bacterial RNA samples. RNA quantity and quality were checked using a NanoDrop (Isogen Life Science, IJsselstein,

The Netherlands) and Experion (Bio-Rad, Veenendaal, The Netherlands), respectively.

Microarray analysis

Synthesis, labeling and hybridization of fungal cDNA to *A. niger* GeneChips were performed according to the instructions of the manufacturer (Affymetrix, 2004). The dsmM_ANIGERa_coll511030F GeneChips are based on the genome sequence of *A. niger* CBS513.88 (Pel *et al.*, 2007) and contain 14 554 probe sets representing all annotated ORFs and genetic elements of *A. niger*. Chip information is available in NCBI's Gene Expression Omnibus (accession number GPL6758). The use of *A. niger* CBS513.88 microarrays for transcriptional profiling of *A. niger* N400, as was done in this study, has a precedent in the literature (van der Veen *et al.*, 2009). *C. fungivorans* Ter331 cDNA synthesis, array hybridization and scanning were performed by NimbleGen (Roche NimbleGen Systems, Iceland). The cDNA was synthesized from 20 µg total RNA and hybridized on a custom-made expression array produced by NimbleGen. Each of the 4480 annotated chromosomal (Leveau *et al.*, 2010; NCBI accession number CP002745) and 43 plasmid-located (Mela *et al.*, 2008; NCBI accession number EU315244) genes was represented by five replicates of 16 60 mers. The array also contained 32 483 control probes used by Nimblegen to quantify background signal and non-specific hybridization. Chip information is available via the MIAMExpress database (accession number A-MEXP-1876).

Data analysis

C. fungivorans microarray data were normalized by NimbleGen using quantile normalization (Bolstad *et al.*, 2003) and the Robust Multichip Average algorithm (Irizarry *et al.*, 2003a, b). *A. niger* microarray data were normalized using the Bioconductor package 'affy' (Gautier *et al.*, 2004). Both data sets were analyzed using the ArrayStar version 2.1.0 (DNASTAR, Madison, WI, USA). To identify genes with significant changes in expression levels, the following criteria were applied: (1) the *P*-value for a moderated *t*-test (Smyth, 2004) with false discovery rate multiple testing correction should be $\leqslant 0.05$ and (2) an absolute fold change in transcript level should be $\geqslant 2$. The fungal and bacterial microarray data have been submitted to the EMBL-EBI's ArrayExpress database under accession number E-MTAB-350.

Validation of microarray data

Validation of microarray data was performed by real-time PCR analysis of a subset of differentially expressed genes. For *Collimonas*, we confirmed the expression of 10 genes that were upregulated based on microarray data and one with unaltered expression. *Aspergillus* microarray results were validated by qPCR analysis on three differentially expressed genes and one unchanged gene. The same RNA used for cDNA synthesis in the microarray experiment was used for reverse transcriptase PCR analysis. Primer pairs (Supplementary Tables S4 and S5) were designed using the software Primer express 3.0 (Applied Biosystems, Warrington, UK). A total of 250 ng of RNA was treated with DNase using a TURBO-DNA-free kit (Applied Biosystems, Nieuwerkerk a/d Ijssel, The Netherlands) and converted into cDNA using the RevertAid H minus first-strand cDNA synthesis kit (Fermentas, St Leon-Rot, Germany). To confirm the absence of genomic DNA contamination, a reaction without the reverse transcriptase was performed for each sample. Each cDNA sample was diluted $5 \times$ in sterile H_2O and 5 µl of the diluted samples were used as template in a 25-µl qPCR reaction containing 100 nM of each primer and $1 \times$ ABsolute QPCR SYBR green PCR master mix (ABgene, Epsom, UK). Reactions were carried out in a Corbett Rotor-Gene 3000 instrument (Westburg, Leusden, The Netherlands), with the following cycling conditions: 95 °C for 15 min followed by 40 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 30 s. The relative standard curve method was applied to estimate gene copy numbers (Applied Biosystems, 2008). Standard curves for each gene were prepared using genomic DNA.

Results and discussion

Visible responses of C. fungivorans and A. niger to each other's presence

In a non-contact confrontation assay, the co-inoculation of *C. fungivorans* Ter331 clearly inhibited the mycelial extension of *A. niger* (Figures 2a and c). Microscopically, we observed deformation and increased branching of the fungal hyphae in the presence of Ter331 (Figure 2b). During mycelial development, the agar became acidic, as indicated by the change in color of the pH indicator bromo-cresol purple (not shown). This acidic halo expanded gradually and ahead of the fungal front, independent of the presence of bacteria. On the confrontation plates, the arrival of the fungal-induced acidification wave coincided with the accumulation of bacterial biomass in the form of slime (Figures 2a and d). In the later stages of the interaction, fungal mycelium turned a darker color, likely as a result of a change in the process of conidiation.

Transcriptional responses of the organisms to each other's presence

Figure 3 shows the numbers of bacterial and fungal genes that were differentially regulated at two time points (T1 and T2) during the confrontation assay. Up to 0.4% of the fungal genome (53 genes) showed altered expression in response to *Collimonas*. The presence of *Aspergillus* also evoked changes in
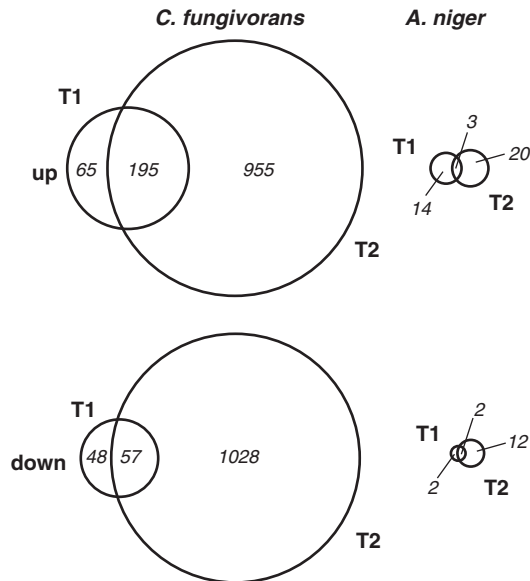
Figure 3 Venn diagrams showing the number of genes differentially expressed in *Collimonas* and *Aspergillus* in the presence of each other at two different time points (T1 and T2). Values represent the number of genes that were ⩾2-fold induced or repressed at the 95% confidence level. Intersects show the numbers of genes that were upregulated or downregulated at both time points. The diameter and overlap of the circles are proportional to the values shown.

the transcriptome of *Collimonas*. At T1, that is at the onset of slime production by the bacteria, 8% (365) of the analyzed genes were differentially expressed at the 95% confidence level. Most of these (71%) were upregulated (Figure 3). At T2, which was 1 day later, as many as 49% of the analyzed genes showed altered expression. Some genes were upregulated at both T1 and T2. Microarray data were validated by quantitative PCR measurements on a subset of regulated genes (Supplementary Figure S1).

Genes differentially expressed in C. fungivorans
Many of the *Collimonas* genes that were upregulated at T1 appeared in distinct clusters on the genome (Figure 4; Supplementary Table S1). A detailed description of these genes is given below. At T2, almost 50% of the *Collimonas* analyzed genes showed an altered transcription level. In contrast with what we observed at T1, the differentially expressed genes were distributed all over the genome.

Carbon and energy metabolism
Cluster A features two genes that code for an oxalate/formate antiporter (Cf_2072 and Cf_2075) and two for a formyl-CoA transferase (Cf_2080 and Cf_2082). The same cluster harbors two genes (Cf_2063 and Cf_2079) with coding homology to several thiamine pyrophosphate-requiring enzymes, including oxalyl-CoA decarboxylases. In the anaerobic bacterium *Oxalobacter formigenes*, which

belongs to the same family as *Collimonas*, the net activity of these activities (that is oxalate in, formate out) generates a proton gradient from which the bacterium derives energy (Anantharam *et al.*, 1989; Dimroth and Schink, 1998). Downstream of Cf_2063 are four genes (Cf_2064-2067) that code for the α-, β-, γ- and δ-subunits of an NAD-dependent formate dehydrogenase (Oh and Bowien, 1998). This enzyme catalyzes the conversion of formate to CO_2 with concomitant production of $NADH_2$, allowing aerobic bacteria such as *Cupriavidus oxalaticus* (Svedruzic *et al.*, 2005) to use oxalate as an energy source. Cluster B encodes several enzymes of the glycerate pathway (Sahin, 2003). This pathway includes tartronic semi-aldehyde reductase (Cf_3540) and glyoxylate carboligase (Cf_3542) and is used by bacteria to assimilate C_2 compounds, including oxalate (Quayle, 1961). Together, these data suggest that in confrontation with *A. niger*, *C. fungivorans* Ter331 is utilizing oxalate as a source of carbon and/or a source of energy. Indeed, oxalate is known to be one of the main acids produced by *A. niger* (Pel *et al.*, 2007), and we could confirm its production by *A. niger* N400 on the confrontation plate by high-performance liquid chromatography analysis of agar extracts (see Materials and methods). In two independent experiments, we measured 0.89 and 1.20 mM oxalate produced by *A. niger* 6 days after inoculation. We could not confirm the ability of *Collimonas* to grow on oxalate as sole source of carbon: in mineral medium, we observed growth to an optical density of ~0.1 or 0.4 with 1 or 5 mM citrate, respectively, but not with oxalate (1 or 5 mM).

Nitrogen metabolism
Several *Collimonas* genes that were upregulated in response to the presence of *A. niger* code for activities that mobilize ammonia. For example, in cluster C, gene Cf_2985 expresses an *fmdA*-like formamidase and Cf_2986 an *amiE*-like acylamide amidohydrolase. FmdA and AmiE release NH_3 from formamide (Wyborn *et al.*, 1996) and aliphatic amides (Skouloubris *et al.*, 2001), respectively. Cluster D contains three genes (Cf_4055-4057) with coding homology to nitrate transporter NasFED (Wu and Stewart, 1998), while cluster E (Cf_224-227) contains *nasCBA*-like genes (Wu and Stewart, 1998) that are involved in the reduction of nitrate to nitrite to NH_3. An additional nitrate transporter is encoded by upregulated gene Cf_223 (also in cluster E). Also upregulated was Cf_556 (cluster F), coding the large chain of glutamate synthase. By itself (that is in the absence of the small chain, which is encoded by Cf_557 and which is not induced in the presence of the fungus), this enzyme exhibits glutaminase activity (Mantsala and Zalkin, 1976), converting glutamine to glutamate and NH_3.

The increased expression of NH_3-mobilizing genes could be a response to an increased C/N ratio and to the induction of bacterial pathways for nitrogen scavenging. This would be consistent with the
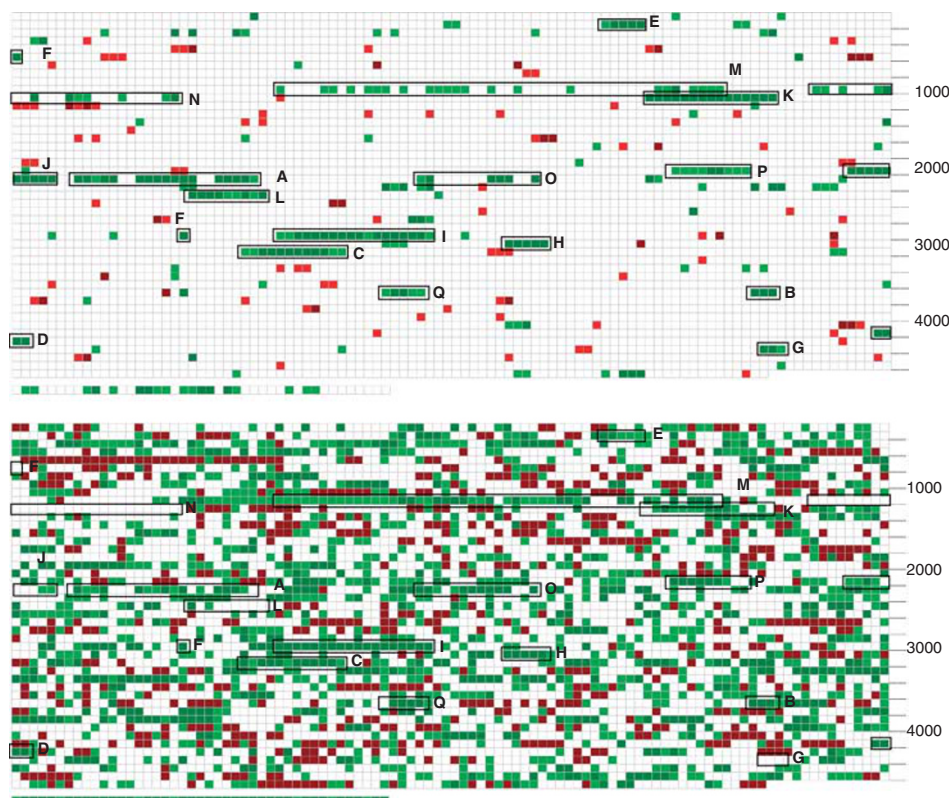
Figure 4 Representation of differentially expressed genes in *C. fungivorans* Ter331 in confrontation with *A. niger* at time points T1 (upper panel) and T2 (lower panel). Each gene is represented by a square in the order as it appears on the genome. The color of a square indicates whether the gene was ⩾2-fold induced (green) or repressed (red), at the 99% (dark green or dark red) or 95% (light green or light red) confidence level. Boxed are gene clusters A–Q that are referred to in the text. In both panels, the bottom row represents genes present on plasmid pTer331.

elevated expression of Cf_2775, which codes for a putative permease for the alternative nitrogen source allantoin. Also upregulated were genes Cf_2912-2916 (in cluster H) and Cf_2987-2991 (in cluster C), both of which code for an UrtABCDE-type transporter of urea (Valladares *et al.*, 2002).

Slime production
Both clusters I (Cf_2786-2803) and J (Cf_2051-2060) contain genes involved in exopolysaccharide production. Exopolysaccharides generate microscale gradients around the cell influencing nutrients concentration, pH and resistance to antimicrobials (Costerton *et al.*, 1987; Stewart, 2002; Stewart and Franklin, 2008). Cluster J comprises 10 upregulated genes, several of which resemble genes coding for the synthesis of colanic acid. In *Escherichia coli*, this polyanionic heteropolysaccharide confers a strong negative charge to the cell surface and offers protection against acidic conditions (Mao *et al.*, 2001). Several genes in cluster I resemble *gum* homologs for xanthan production in *Xanthomonas campestris* (Becker and Vorhölter, 2009). Xanthan synthesis is increased by exposure of *X. campestris* to acid stress (Roseiro *et al.*, 1996). We tested experimentally whether exposure of *Collimonas* to

an acidic environment triggers the production of slime (Supplementary Figure S2). Both HCl and oxalate acidified the agar but did not induce slime production. Citrate induced the production of slime, but did so also at a non-acidifying pH of 6.5. Slime production was further observed with *N*-acetylglu-cosamine, glycerol and glucose, but not with water. From these experiments, we conclude that (1) slime production requires a carbon source and (2) low pH is not required for slime production to occur. Another possible motivation for slime production is the dissipation of excess carbon. This was observed for *Xanthomonas* in the presence of organic acids and absence of growth (Esgalhado *et al.*, 2001).

Secondary metabolites and bacterial secretion
Cluster K (Cf_1128-1142) encompasses 15 upregulated genes, some resembling polyketide synthases and non-ribosomal peptide synthetases. We have preliminary evidence that these genes code for the synthesis of a compound with antifungal activity that is responsible for the inhibition of fungal growth and deformation of fungal hyphae (Figures 2a–c). Identification and characterization of this compound will be the subject of a separate study

(Fritsche *et al.*, in preparation). Cluster L (Cf_2276-2284) codes genes belonging to a type II secretion system, a pilus-like structure specialized in transporting toxins and hydrolytic enzymes in the extracellular space (Cianciotto, 2005; Beeckman and Vanrompay, 2009).

Motility
Cluster M (Cf_986-1036) contains 23 upregulated genes related to flagellum synthesis and chemotaxis, suggesting that *Collimonas* is trying to get away from the fungus or move towards it. Our confrontation plates contained 2% agar, which typically does not allow flagella-driven motility. The possession of flagellum synthesis genes is in agreement with the observation by electron microscopy of polar flagella (de Boer *et al.*, 2004).

Horizontal gene pool
Many of the genes located on plasmid pTer331 (Mela *et al.*, 2008) showed increased expression in response to the fungus. The same was true for genes in clusters N (Cf_1047-1074) and O (Cf_2102-2115), both of which carry genes belonging to putative prophages. The activation of mobile genetic elements is a common feature of bacterial response to stress (Foster, 2007).

General stress response and ribosomal proteins
In total, 57 genes were significantly downregulated at both T1 and T2 (Figure 3). Closer examination of these revealed a disproportionate number of genes coding for ribosomal proteins. Ribosomal proteins are essential for *de novo* synthesis of proteins (Brodersen and Nissen, 2005; Wilson and Nierhaus, 2005). Their downregulation has been linked to various stress experiences (Ishige *et al.*, 2003; Lawrence *et al.*, 2004; Silberbach *et al.*, 2005; Silberbach and Burkovski, 2006) and a decrease of cellular growth (Stintzi, 2003). Genes Cf_3502 and Cf_2039, in clusters P and Q, respectively, encode two Csb proteins (Akbar *et al.*, 1999; Prágai and Harwood, 2002). These are activated during the general stress response, which is triggered by energy or environmental stress and aims at protecting DNA, proteins and membranes from damage (Hecker and Volker, 1998). Consistent with this, genes Cf_2035 and Cf_2036, in cluster P, encode two ATP-dependent DNA ligase, involved in repairing DNA double-strand breaks (Aravind and Koonin, 2001).

Genes differentially expressed in *A. niger*
As for the fungal genes that were differentially expressed in response to the presence of *Collimonas* (Figure 3; Supplementary Tables S2 and S3), their putative functions collectively suggest the fungal experience of (nutrient) stress and the need for rearrangement of cell walls and cell membranes.

Nitrogen metabolism
The differential expression of several *Aspergillus* genes suggests that in confrontation with *Collimonas*, the fungus experienced a shortage of nitrogen. One of the downregulated genes, An01g11380, codes for a homolog of NmrA, which is involved in controlling nitrogen metabolite repression in various fungi (Stammers *et al.*, 2001). In *Aspergillus nidulans*, a *nmrA* deletion partially derepressed the utilization of non-preferred nitrogen sources (Andrianopoulos *et al.*, 1998). Consistent with this, gene An02g00560, which codes for a uric acid-xanthine transporter and is normally repressed by NmrA (Gorfinkiel *et al.*, 1993; Stammers *et al.*, 2001) was upregulated at T2. Three other genes that were induced at T1, that is An07g00370, An07g08770 and An13g03910 (also induced at T2), show coding homology to proteins involved in the transport and metabolism of the alternative nitrogen source allantoin/allantoate. Three of the 10 most highly upregulated genes in the fungus at T1 (that is An01g14550, An18g01740 and An10g00730) are coding for nitrilases, suggesting that nitriles may also serve as sources of nitrogen for the fungus.

Stress response
Three genes that were downregulated at T2, that is An06g00900, An06g00930 and An06g00940, are located near each other on the genome. Of these, An06g00940 was annotated as NUCB1, a conserved regulatory protein (de Alba and Tjandra, 2004). One of its proposed functions is to suppress the endoplasmic reticulum stress response, which is activated under conditions that lead to malfunctioning of the endoplasmic reticulum and unfolding/misfolding of proteins (Tsukumo *et al.*, 2007). Downregulation of this repressor suggests that such stress conditions are met in confrontation with *Collimonas*. Upregulated at T2, gene An01g10790 is a *con-10* homolog, typically expressed during conidial differentiation (Roberts *et al.*, 1988; Corrochano *et al.*, 1995), which might be another indication that exposure to *Collimonas* induces fungal stress.

Cell membrane
Several of the differentially expressed genes in *A. niger* are linked to the fungal cell membrane. Two genes, both upregulated at T2, are involved in regulating cell membrane fluidity. The first, An12g09940, resembles the *sdeA* gene of *A. nidulans* (Wilson *et al.*, 2004), which encodes a Δ9-stearic acid desaturase for the production of unsaturated fatty acids. The second, An03g00580, codes for a putative CypX-like cytochrome P-450 (van den Brink *et al.*, 1998). The latter is a key enzyme in the biosynthesis of ergosterol, which is required for membrane permeability and fluidity. Upregulation of An12g09940 and An03g00580

suggests an effort by the fungus to regulate membrane fluidity, which in *Candida albicans* and *Aspergillus fumigatus* was suggested to confer resistance to amphotericin B (Hamilton, 1972; Gautam *et al.*, 2008). This polyene antifungal agent binds to ergosterol to form pores that cause leakage of hyphal content and eventually death of the fungus (Moore *et al.*, 2000). An16g05910 and An16g05920 are adjacent genes, both downregulated in our experiment. While An16g05920 codes for a putative membrane receptor of a yet unidentified signal (De Zwaan *et al.*, 1999), An16g05910 is homologous to gene *ahbB1* of *A. nidulans*, which is believed to be involved in cell membrane synthesis (van den Brink *et al.*, 1998). Its deletion in *A. nidulans* led to an increased branched phenotype (Lin and Momany, 2004), which closely resembled the hyphal deformations that we observed for *A. niger* in confrontation with *Collimonas* (Figure 2b).

Cell wall

The function of An12g10200, upregulated at T1, is unknown, but it was shown to be induced upon exposure of *A. niger* to caspofungin (Meyer *et al.*, 2007), an antibiotic that inhibits the synthesis of the fungal cell wall component β-1,3-glucan. Another cell-wall-related gene was downregulated at T2, that is An08g09420, coding for galactomannoprotein (Pel *et al.*, 2007), a structural component of the *Aspergillus* cell wall (Yuen *et al.*, 2001). Also downregulated was An09g06400, a homolog of the *A. nidulans chiA* gene. Its product functions as a chitinase involved in cell wall remodeling and/or maturation (Yamazaki *et al.*, 2008). During branching of *A. niger* hyphae, gene An09g06400 was downregulated (Meyer *et al.*, 2009). Differential regulation of these genes suggests that the presence of *Collimonas* promotes changes in fungal cell wall dynamics, some of which are likely to be linked to the changes in hyphal morphology (Figure 2b).

A working model for the *Collimonas/Aspergillus* interaction

Figure 5 presents a summary of the confrontation between *C. fungivorans* Ter331 and *A. niger* N400, as deduced from the transcriptional profiles of both organisms. It is clear that the interaction is quite complex, and resembles a back-and-forth dialog between the two partners.

One key component of the working model is our demonstration that oxalate is produced by the fungus on confrontation plates and that genes involved in the uptake and metabolism of oxalate were upregulated in *Collimonas*. We were however not able to confirm the ability of *Collimonas* to use oxalate as a source of carbon, nor did the bacterium produce slime in response to oxalate. Thus, under the confrontation conditions, oxalate might not represent a source of fungal-derived carbon for the bacterium, but instead a source of fungal-derived energy. This invites the interesting question whether or not the original definition of bacterial mycophagy (that is the conversion of fungal biomass into bacterial biomass; Leveau and Preston, 2008) should be broadened to include the conversion of fungal biomass into energy available to bacteria. If *Collimonas* is not able to use oxalate as a source of carbon, then the production of slime as observed in the confrontation assay cannot be explained by the fungal release of oxalate. Indeed, we could show that slime production also occurred in confrontation of *Collimonas* with a mutant derivative of *A. niger* N400, which is not able to produce oxalate (Supplementary Figure S3). This mutant however did acidify the medium (Supplementary Figure S3), suggesting the release of organic acids other than oxalate. Confrontation of *Collimonas* with another *Aspergillus* strain, *A. westerdijkiae*, also resulted in slime production, but without acidification of the agar (Supplementary Figure S3). Combined, these observations suggest that *Collimonas* mycophagy (defined in this assay as the accumulation of bacterial biomass in response to fungally released compounds) appears to not be exclusively dependent on oxalate as an energy source or organic acids as a carbon source. This possibility will be tested in future studies.

It is intriguing that *Collimonas* genes for chemotactic motility (cluster M) were upregulated. Bacterial attraction to fungally produced compounds, including organic acids has been reported previously. For example, *Pseudomonas fluorescens* is chemoattracted to fusaric acid produced by *Fusarium oxysporum* f. sp. *radicis lycopersici* (Forl) (de Weert *et al.*, 2004). Of particular interest to us is oxalate, given the upregulation of *Collimonas* genes involved in the utilization of this compound. Secretion of oxalate is a common trait among fungi (Dutton and Evans, 1996), but also plants (Horner and Wagner, 1995). It is worth noting that several collimonads known to date were isolated from the ectomycorrhizae of pine trees (Leveau *et al.*, 2010), which produce oxalate as a weathering agent to release phosphate and other micronutrients from the soil environment (Rosling, 2009). *Collimonas* was also detected on the hyphae of *Resinicium bicolor* (Folman *et al.*, 2008), a fungus that is known to accumulate oxalate crystals on its surface. These observations suggest a possible role for oxalic acid in the attraction of *Collimonas* to fungi.

Both partners in the *Collimonas/Aspergillus* confrontation experienced nitrogen deficiency in each other's presence. This indicates competition for a limited resource, which adds an additional level of complexity to the interaction. It remains to be determined how this competition contributed to the response of both organisms to each other's presence. It is possible that the sequestration of NH_3 by *Collimonas*, as suggested by its transcriptional
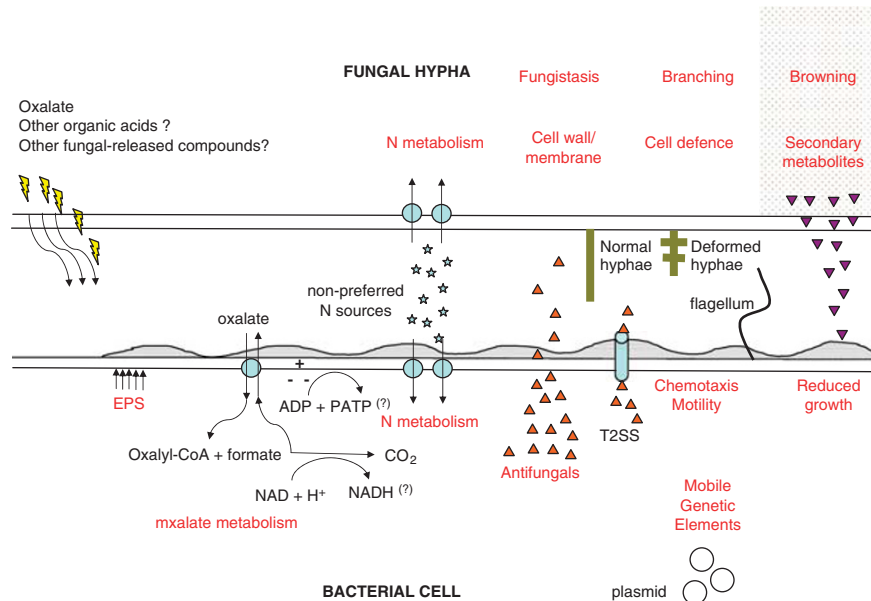
Figure 5 Schematic overview of the *Collimonas/Aspergillus* dialog, as interpreted from transcriptional profiling of both partners. Details are discussed in the text.

profile, exacerbated the nitrogen shortage experienced by *A. niger*. Other types of stresses were noted as well, for example acid stress in the bacteria and endoplasmic reticulum stress in *A. niger*. How 'natural' these responses are is uncertain. Obviously, the arena in which we confronted the bacterium and fungus lacked the complexities and dynamics of real-life environments. However, the transcriptional profiles clearly offered insight into the functional hardwiring of both microorganisms, which allows them to survive in their natural habitats. The *Collimonas* data showed concurrent expression of genes involved in mycophagy, nutrient deficiency, low pH stress and antibiosis, which fits well with its description in the literature as a soil bacterium with antifungal activity and that prefers acidic, nutrient-poor environments (Leveau *et al.*, 2010).

Acknowledgements

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