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Fungus-associated bacteriome in charge of their host behavior

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ABSTRACT

Bacterial-fungal interactions are widespread in nature and there is a growing number of studies reporting distinct fungus-associated bacteria. However, little is known so far about how shifts in the fungus-associated bacteriome will affect the fungal host's lifestyle. In the present study, we describe for the first time the bacterial community associated with the saprotrophic fungus *Mucor hiemalis*, commonly found in soil and rhizosphere. Two broad-spectrum antibiotics that strongly altered the bacterial community associated with the fungus were applied. Our results revealed that the antibiotic treatment did not significantly reduce the amount of bacteria associated to the fungus but rather changed the community composition by shifting from initially dominating *Alpha-Proteobacteria* to dominance of *Gamma-Proteobacteria*. A novel approach was applied for the isolation of fungal-associated bacteria which also revealed differences between bacterial isolates obtained from the original and the antibiotic-treated *M. hiemalis*. The shift in the composition of the fungal-associated bacterial community led to significantly reduced fungal growth, changes in fungal morphology, behavior and secondary-metabolites production. Furthermore, our results showed that the antibiotic-treated isolate was more attractive and susceptible to mycophagous bacteria as compared to the original isolate. Overall, our study highlights the importance of the fungus-associated bacteriome for the host's lifestyle and interactions and indicate that isolation with antibacterials is not sufficient to eradicate the associated bacteria.

1. Introduction

Bacteria and fungi are widespread in nature and play important roles in many ecological processes. Similar to other organisms, many fungi have an associated bacteriome and there is a growing number of known endosymbionts where bacteria dwell within fungal hyphae (Bonfante and Anca, 2009; Kobayashi and Crouch, 2009). Another type of fungal-bacterial interaction is ectosymbiosis, where the bacterial partner is adhered to fungal hyphae (Stopnisek et al., 2016; Warmink et al., 2009).

The symbioses with ecto- and endofungal bacteria are often overlooked, yet they may have a profound effect on the fungus behavior and lifestyle. For example, in *Aspergillus nidulans* and *A. niger*, the ectobacteria actinomycetes and *Bacillus subtillus* respectively, were shown to affect fungal primary and secondary metabolism (Benoit et al., 2015; Schroeckh et al., 2009).

It is plausible that there is a relationship between fungi and bacteria of which both partner profit. For example, bacteria or fungi can benefit from specific compounds that are produced by the other partner if they cannot produce it themselves. Several mycorrhizal helper bacteria secrete citric and malic acids that are metabolized by *Laccaria bicolor*, promoting its growth (Duponnois and Garbaye, 1990). Conversely, ectomycorrhizal fungi may produce organic acids or sugars that can affect the composition and growth of associated bacterial communities. For example, the helper bacterial isolate *P. fluorescens* BBc6R8 can be chemoattracted by the hyphae of the ectomycorrhizal fungus *L. bicolor* S238N and the trehalose accumulated within the mycelium that promotes the growth of the helper bacterium (Deveau et al.,

Abbreviations: ARE, artificial root exudates; CFU, colony forming units; MA, antibiotic-treated *M. hiemalis* isolate; M0, original *Mucor hiermalis* isolate; OTU, Operational Taxonomic Unit; PDA, potato dextrose agar; TSB, Tryptic Soya Broth; VOCs, volatile organic compounds; WA, water agar; WYA, Water Yeast Agar.

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2010). In the interaction between *S. cerevisiae* and several *Acinetobacter* species, ethanol secreted by the yeast was shown to stimulate the growth of the bacterial species and it can act as a signaling molecule, altering cell physiology (Smith et al., 2004).

Bacterial endosymbionts *Candidatus Glomeribacter gigasporarum* of arbuscular mycorrhizal fungi *Gigaspora margarita* can be involved in the vitamin B12 provision for the fungus (Ghignone et al., 2012). The co-adaptation between fungal hosts and bacterial endosymbionts can become so tight that the genome size and the gene content of the endosymbionts is reduced. Mycorrhizal endosymbionts from Mollicutes lineages were suggested to depend metabolically on their host, and additionally have taken up regulatory eukaryotic genes horizontally (Naito et al., 2015). Clear evidence for gene transfer between Mollicute-related endobacteria and their mycorrihizal host *Dentiscutata heterogamma* was recently reported (Torres-Cortes et al., 2015). Furthermore, endobacteria can improve the fitness of their host by e.g. increasing the fungal sporulation success and raising the fungal bioenergetics capacity (Salvioli et al., 2016). Another well-studied example is the rice seedling blight pathogen *Rhizopus microsporus* (Lackner et al., 2009; Partida-Martinez and Hertweck, 2005). This fungus contains endobacteria named *Burkholderia rhizoxinica* and *Burkholderia endofungorum* (Partida-Martinez et al., 2007a), which have been shown to produce a potent toxin involved in host pathogenesis (Gee et al., 2011; Partida-Martinez and Hertweck, 2005). Interestingly, the endobacteria enforce their vertical transmission by controlling host sporulation making use of a hrp type III secretion system (Lackner et al., 2011; Partida-Martinez et al., 2007b). As a result, the host is not able to reproduce in absence of its endosymbiont, thereby ensuring maintenance of the symbiosis (Partida-Martinez et al., 2007b).

Microscopic and molecular analysis showed that several nitrous oxide-producing fungal isolates of *Mortierella elongata* harboured endobacteria in their mycelia (Sato et al., 2010). The sequencing of 16S rRNA genes revealed that the N_2O-producing fungus *Mortierella elongate* harbored endobacteria belonging to the family *Burkholderiacea*, however, the significance of this fungal-bacterial association is unknown (Sato et al., 2010). Recently, an endophyphal bacterium living in association with *Mortierella elongate* was isolated and on the basis of phenotypic, chemotaxonomic and pylogenetic characteristics it was identified as a novel genus and species, for which the name *Mycoavidus cysteinexigens* gen. nov., sp., nov was proposed (Ohshima et al., 2016).

It is a common practice prior performing experiments with soilborne fungi to pre-culture them on a media supplemented with antibiotics (Ballhausen et al., 2016; Singh et al., 2015). In our lab working with the saprotrophic fungus *Mucor hiemalis* (Zygomycota) isolated from the rhizosphere of *Carex arenaria* (sand sedge) (De Rooij-van der Goes et al., 1995), we observed that treatment with broad-spectrum antibiotics strongly altered the fungal morphology and hyphal extension. Based on this observation, we aimed first to determine the bacterial community associated with *Mucor hiemalis* and to test if the antibiotic treatment resulted in bacteria-free fungus. Furthermore, we aimed to reveal how changes in the bacterial community affect fungal fitness, behavior, metabolites production and interactions.

2. Material and methods

2.1. Fungal strains and growth conditions

The *Mucor hiemalis* isolate M0 obtained from the rhizosphere of *Carex arenaria* (Sand sedge) collected from sandy dune soil in the Netherlands was originally isolated on malt extract agar supplemented with 50 ppm oxytetracycline (De Rooij-van der Goes et al., 1995). Here, the antibiotic-treated isolate MA derived from the isolate M0, after plating on King's B agar with antibiotics (rifampicin and kanamycin 50 mg/ml, final concentration), and transferred to Water Yeast Agar (WYA) (Garbeva et al., 2011) and Oatmeal agar OA (24 g/L Difco, France) supplemented with rifampicin and kanamycin (50 mg/ml final concentration). The spores were washed in rifampicin and kanamycin solution (50 mg/ml final concentration) and collected over glass wool with sterile deionized water and stored at -80 °C. Spores of the M0 and MA isolates were grown on nutrient-rich 0.5 strength Potato dextrose agar (PDA, Oxoid, England; pH 6), and nutrient-poor Water agar + $(NH_4)_2SO_4$, pH 6.7 (Garbeva et al., 2011).

2.2. Fungal identification

The identity of the isolate M0 and MA was confirmed using the ITS sequences targeted by the primers ITS1 and ITS4 (White et al., 1990). Fungal DNA was extracted using the ZR Fungal/Bacterial DNA MiniPrep (Zymo Research, USA) according to the manufacturer's instructions. Extracted DNA was used for PCR amplification with a master mix containing 1x FastStart High Fidelity Reaction Buffer (Roche) with 18 mM $MgCl_2$ (Roche), 0.04 U FastStart High Fidelity Enzyme Blend (Roche), 200 µM of each dNTP, 0.6 µM ITS1 and ITS4 primer. The thermal protocol was as followes: initial denaturation at 95 °C for 5 min, and 35 cycles of 95 °C for 30 s, 55 °C for 30 s, 72 °C for 1 min, and a final elongation at 72 °C for 10 min. The PCR product was cleaned using a PCR purification kit (QIAGEN Benelux B.V., The Netherlands) and sent for Sanger sequencing to Macrogen Europe (Amsterdam, The Netherlands). Obtained sequences were checked for quality using BioEdit (Hall, 1999) and identified using NCBI nucleotide database BLASTn (Altschul et al., 1990).

2.3. Hyphal extension of fungal isolates M0 and MA

For measuring hyphal extension, 6-mm-diameter agar disks taken from the edge of the fungal hyphae of M0 or MA (pregrown on 0.5 strength PDA) were plated in the middle of nutrient-rich 0.5 strength PDA or nutrient-poor WA plates (8.5 cm diameter). Per fungal isolate and agar type (i.e. PDA and WA) three plates were set-up. The plates were sealed with parafilm and incubated at 20 °C for 5 days. On the fifth day, for each plate the extension of fungal hyphae was measured with a ruler in three coordinates and the average extension was calculates.

2.4. Competition assay of fungal isolates M0 and MA

Two plugs taken from the edge of the (on 0.5 strength PDA) pregrown fungal hyphae of M0 or MA were placed with 3 cm distance from each other on a new 0.5 strength PDA plate (8.5 cm diameter) and incubated at 20 °C. As control, both fungal isolates were incubated separately on 0.5 strength PDA at the same time. For each treatment three replicates were set-up. The hyphal extension was monitored at day 7, 14 and 21 after inoculation. Pictures were taken with a Panasonic DMC-FZ200 digital camera. The area of M0 and MA hyphal extension was measured with AxioVision V 4.9.1.0 (Carl Zeiss Microscopy GmbH, Germany). A relative area for the hyphal extension per replicate was calculated by dividing the area obtained in the competition experiment per fungal isolate by the average area obtained for each control.

2.5. Bioassay for testing the fungal growth inhibition by bacterial secondary metabolites

The bacterial strains *Burkholderia* sp. AD024 (De Ridder-Duine et al., 2005; Schulz-Bohm et al., 2015) and *Collimonas fungivorans*