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Endofungal Bacteria Increase Fitness of their Host Fungi and Impact their Association with Crop Plants

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Abstract:
Endofungal bacteria are bacterial symbionts of fungi that exist within fungal hyphae and spores. There is increasing evidence that these bacteria, alone or in combination with their fungal hosts play a critical role in tripartite symbioses with plants, where they may contribute to plant growth and disease resistance to microbial pathogens. As the frequency of bacteria in fungi is commonly very low, breakthroughs in technology such as molecular taxonomy and laser scanning microscopy were required to establish the functional contribution of these bacteria in complex symbioses. Yet, the overall biological significance of endofungal bacteria is largely unknown and further progress in understanding is hampered by a very few biological systems where endofungal bacteria have been described mechanistically. We review here the current knowledge on endobacteria (EB) and their role in different types of fungal symbioses with plants. We show that various attempts to cure fungal cells from endobacteria failed, further suggesting that they play a crucial role in the symbiosis. Moreover, isolation of some of the endobacteria from their fungal hosts allowed confirming their autonomous beneficial activity such as plant growth promotion and resistance-inducing activity. The review addresses the potential agricultural significance of endofungal bacteria and their role in supporting sustainable agriculture by promoting plant growth, improving plant resistance, and decreasing yield loss caused by many microbial pathogens.

Endobacteria in plant-colonizing fungi

Endofungal bacteria inhabit the cytoplasm of fungal cells (Figure 1). They commonly establish beneficial relationships (positive symbioses) with their plant-colonizing host fungi thereby forming tripartite interactions that comprise the bacterium, the fungus and the plant (Perotto and Bonfante, 1997; Bonfante and Anca, 2009; Desirò et al., 2014; Moebius et al., 2014; Erlacher et al., 2015; Glaeser et al., 2016; Salvioli et al., 2016). From the historical perspective, Mosse (1970) was the first to describe intracellular structures very similar to bacteria, called Bacteria-Like Organisms (BLOs) inside fungal hyphae (Figure 2). Since then, BLOs and bacteria were detected in glomeromycotan arbuscular mycorrhiza

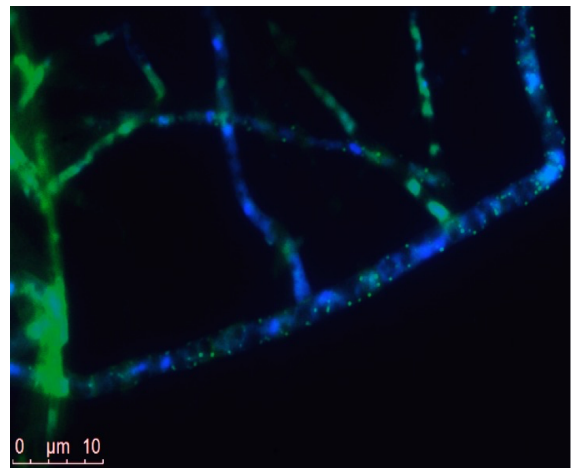


Figure 1. Detection of endofungal bacteria in fixed fungal mycelia of *Sebacina vermifera*-MAFF305838 by Fluorescence *in situ* hybridization (FISH) analysis using a universal Bacteria 16S rRNA targeting probe (green) and DAPI counter straining (blue). Detection of endobacteria in this strain was first described in Sharma et al. (2008).

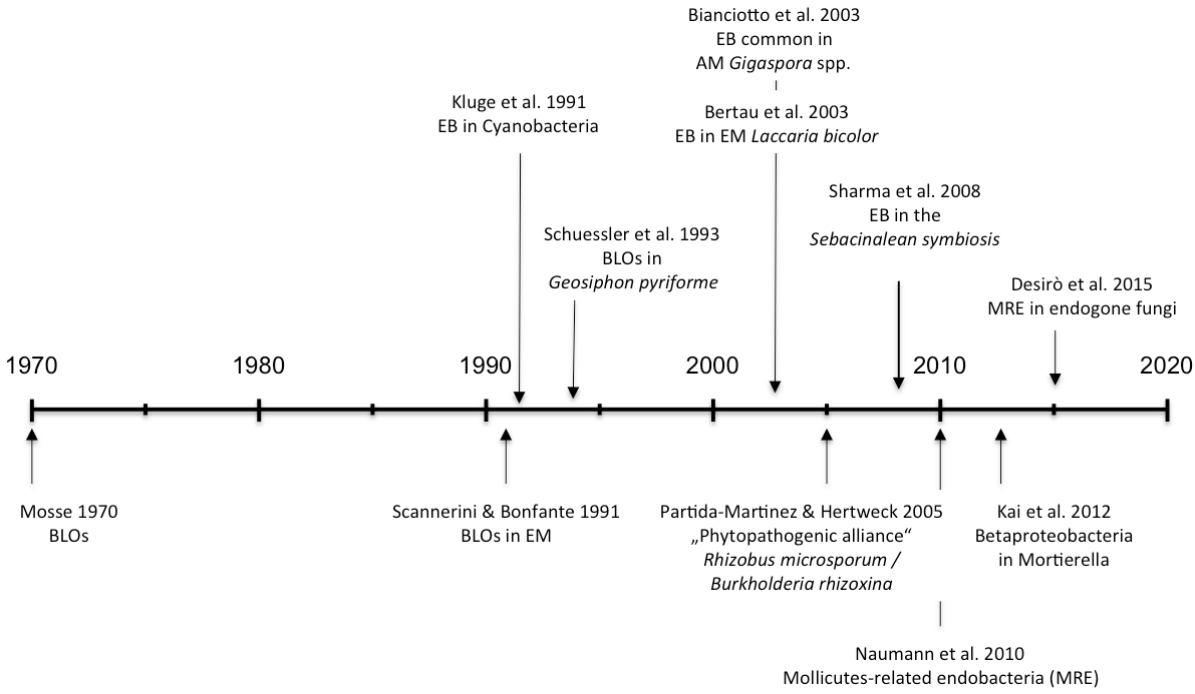
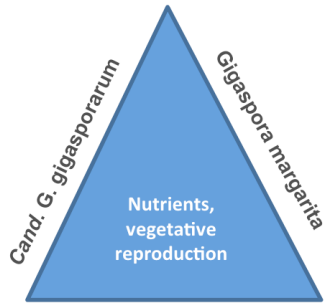


Figure 2. Time scale of the published research on endofungal bacteria.

(AM) symbioses (Scannerini and Bonfante, 1991; Bianciotto et al., 2003; Naumann et al., 2010), the related fungus *Geosiphon pyriforme* (Kluge, 1992; Schüßler et al., 1994), the ectomycorrhizal (EM) basidiomycete fungus *Laccaria bicolor* (Bertaux et al., 2003; 2005), the rice pathogenic fungus *Rhizopus microsporus* (Partida-Martinez and Hertweck, 2005), phylogenetically diverse foliar fungal endophytes (Hoffman and Arnold, 2010), and the plant symbiotic Endogone Mucoromycotina fungi (Desirò et al., 2015). Unlike insect endosymbionts, which are localized in specialized tissues (Moran et al., 2008), endofungal bacteria are found in fungal spores and both extra- and intraradical hyphae, the latter colonizing the plant tissues (Lumini et al., 2007). Endofungal bacteria also have been discovered in the Sebacinalean symbiosis, a mutualistic association of Basidiomycota fungi of the order Sebacinales with a broad spectrum of plants (Sharma et al., 2008). Although the role of these bacteria is not fully understood, increasing evidence gathered by studying the model Sebacinalean fungus *Piriformospora indica* suggests that they contribute to the fitness of their fungal hosts (Guo et al., submitted). Some reports illustrate that endofungal bacteria support the virulence of pathogenic fungi (Scherlach et al., 2006). In general, complex interactions including bacteria, fungi, and plants are poorly understood, and the global prevalence of these types of interactions is largely unknown. In this review we focus on the three most intensively studied biological models comprising endobacteria in beneficial and parasitic tripartite interactions (Figure 3).

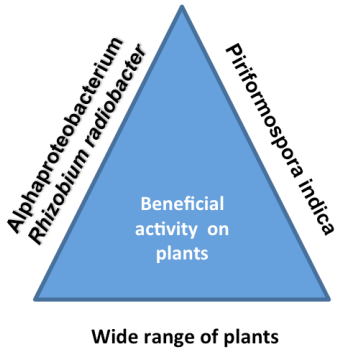
Endofungal bacteria in the AM symbiosis

Two types of endofungal bacteria have been distinguished in AM fungi on the basis of their morphological features. The first type of endobacteria is coccoid-shape and present also in the cytoplasm of another basal group of fungi, the Endogone Mucoromycotina species. These bacteria represent a monophyletic clade of fungal mycelium-derived sequences which is phylogenetically placed in-between the Mollicutes and Firmicutes and termed Mollicutes-related endobacteria (MRE; Scannerini and Bonfante, 1991; Naumann et al., 2010; Desirò et al., 2015). In contrast to the cell-wall free Mollicutes, the MRE endobacteria detected in the cytoplasm of fungal cells contain a Gram-positive like cell wall.



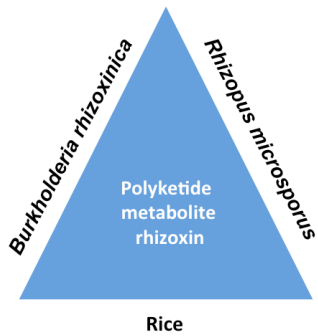
Model association for AM symbiosis:
Cand.* Glomeribacter gigasporarum / *Gigaspora margarita
Bonfante et al. 2003

- **Bacterial genome reduced**
- **Axenic culturing of bacteria very artificial**
- **Cured fungus compromised in vegetative reproduction**
- **EB surrounded by host plasma membrane**



Model association for Sebacinalean symbiosis:
Rhizobium radiobacter / Piriformospora indica
Sharma et al. 2008; Glaeser et al. 2016)

- **Bacterial genome not reduced but specialized**
- **Axenic culturing of bacteria possible**
- **Partially-cured fungus reduced in vegetative reproduction**
- **Pure EB exhibit beneficial activity on host plants**



Model association for a plant pathogenic interaction:
***Burkholderia rhizoxinica* / *Rhizopus microsporus* / Rice**
(Partida-Martinez & Hertweck 2005)

- **Bacterial genome not reduced but specialized**
- **Axenic culturing of bacteria possible**
- **Cured fungus compromised in vegetative reproduction**
- **EB produce phytotoxin**

Figure 3. Comparative summary of the knowledge about the three most extensively studied tripartite symbioses of plant with their colonizing fungi and endobacteria.

The second type of endofungal bacteria are Gram-negative rod-shaped bacteria that are restricted to the AM-forming family *Gigasporaceae* (Bianciotto et al., 2004). Those intracellular bacteria were detected in five *Gigaspora* spp. through all stages of the fungal life cycle: spores, germtubes, extra- and intraradical hyphae. Based on the 16S rRNA gene sequence phylogeny, the endobacteria in *Gigaspora margarita* were initially identified as a member of the genus *Burkholderia* (Bianciotto et al., 1996). The *G. margarita* isolate BEG 34 contained 250,000 living bacterial cells in a single spore. The bacteria were subsequently classified based on genetic features as a novel bacterial taxon, *Candidatus* Glomeribacter gigasporarum (*Ca*Gg; Banciotto et al., 2003) next closest related to the genus *Burkholderia*. Isolated bacteria could not be grown in culture media, but kept alive for up to 4 weeks

(Jargeat et al., 2004). Sequencing of the genome of a homogeneous cell population derived from the *Gigaspora margarita* strain BEG 34 representing *Ca*Gg led to a 1.72 Mb assembly with 1,736 coding DNA sequences (CDS), the smallest genome known for a Betaproteobacterium (Ghignone et al., 2012). Such small genomes are typically found in endocellular bacteria living permanently in their host with an obligate symbiotic lifestyle, where gene erosion occured in association with metabolic dependence on the host (Moran et al*.,* 2008; Castillo and Pawlowska, 2010). The genome assembly comprised one chromosome and three plasmids. Although the genome was rather small, the G+C content was high (54.8%), which is unusual for small genomes.

A phylogenetic multilocus gene analysis carried out on concatenated sequences of 21 protein-coding genes retrieved from 67 completed genomes belonging to a wide range of bacterial lineages showed that *Ca*Gg clusters within the family *Burkholderiaceae*. Members of this family are highly versatile microbes interacting with animals, humans, plants and fungi (Bontemps et al., 2010). Analysis of a data set restricted to *Betaproteobacteria* and based on 16S and 23S rRNA gene sequences placed *Ca*Gg as a sister group of a *Burkholderia* clade, which includes free-living and fungal-associated species showing that *Ca*Gg is an ancient member of the taxon sharing a common ancestor with the present-day *Burkholderiaceae* (Ghignone et al., 2012). While phylogenetic analyses placed *Ca*Gg in the *Burkholderiaceae*, metabolic pathway analyses clustered it with endosymbiotic bacteria of insects. This positioning among different bacterial classes let the authors claim that *Ca*Gg has undergone convergent evolution to adapt itself to an intracellular lifestyle. Genome annotation further revealed an unexpected genetic mosaic where typical genetic determinants of symbiotic, pathogenic and free living bacteria are integrated in a reduced genome.

Significantly, the bacterial cells are separated from the fungal cytoplasm by a membrane of fungal origin, suggesting that *Ca*Gg communicates with the fungus via transport and secretion systems that deliver bacterial molecules to the host. This is supported by the large number of genes present in its genome coding for secretion, including type II and type III secretion systems and synthesizes vitamin B12, antibiotics- and toxin-resistance molecules, which may contribute to the fungal host's ecological fitness (Ghignone et al., 2012). On the other hand, genome annotation also provided an insight into the molecular basis for *Ca*Gg obligate biotrophic status: The lack of some crucial metabolic pathways can explain the failure to grow *Ca*Gg as a free-living organism, as it has a metabolic dependence on the fungal host for both energy and nutrition. Thus, the bacterial genome provided clear evidence of the energy/nutrient flows of the tripartite interaction between the bacterium, the fungus and the plant. *Ca*Gg*'s* limited capacity to synthesize amino acids, the presence of a large set of amino-acid transporters in the bacterial genome, and its location inside the protein-rich fungal vacuoles highlight that there is most likely a flow of nitrogen from the fungus to the bacterium. Consistent with the strong dependency, *Ca*Gg is at least in part but non-essential vertically transmitted through fungal vegetative sporulation, indicating that active bacterial proliferation occurs in the multinucleate mycelium of the fungus (Bianciotto et al*.,* 2004). Consistent with this, the genome evolution in *Ca*Gg is non-degenerative and exemplifies a departure from the model of degenerative evolution in heritable endosymbionts such as mutualists of insects (Mondo et al., 2016).

While the occurrence of *Ca*Gg is limited to the *Gigasporaceae*, coccoid-shaped MRE endobacteria are widely distributed across different lineages of AM fungi. A fungus can harbor both types of endobacteria, with MRE population being more abundant, variable and prone to recombination (Desirò et al., 2014), suggesting that *Gigasporaceae* with their comparatively large spores, which are rich in reserves of glycogen, fats and proteins, can support the energetic cost of complex bacterial communities. MRE were also identified in the Endogone Mucoromycotina fungi, an ancient group of fungi capable of symbiotically interacting with plants. Interestingly, Mucoromycotina fungi along with Glomeromycota are considered as the unique ancestral symbionts of land plants (Desirò et al., 2015). MRE possess a homogenous cell wall-like envelope, which upon high pressure and freeze substitution is rather electron-transparent. 16S rRNA gene sequences of MRE isolated from AM fungi and Endogone Mucoromycotina fungi cluster together and form a separate clade. In both fungal groups, coccoid-shaped MRE are embedded in the fungal cytoplasm without any evidence of the fungal membrane which surrounds *Ca*Gg (Ghignone et al., 2012).

Endofungal bacteria impact the AM symbiosis

The mechanistic role of AM-associated endobacteria is still unclear, but their presence may be required for successful interaction of the fungi with their plant hosts. *In vitro* studies suggested that *Paenibacillus* sp. may stimulate AM's hyphal growth (Horii and Ishii 2006; Horii *et al*. 2008), the formation of new spores (Hildebrandt et al., 2002), and the suppression of pathogens (Budi et al., 1999; Horii et al., 2008). Though these bacteria were associated with the mycorrhizosphere, a true endofungal lifestyle has not been proven yet. Similarly, when surface-sterilized spores of *Glomus intraradices* Sy167 were germinated on agar plates, slime-forming bacteria, identified as *Paenibacillus validus*, frequently grew up. These bacteria supported the fungus completing its life cycle in the absence of plant roots (Hildebrandt et al., 2006).

Clear evidence for endofungal bacteria essentially contributing to beneficial AM symbioses is still missing because killing or separating bacteria from their AM host by antibiotic treatments or single spore proliferation is extremely difficult. The reason for this intimate association between endofungal bacteria and their hosts is not exactly known and may vary for single interaction systems. Yet, *G. margarita* BEG 34 provides a convincing example for a cured fungus: repeated passages through single-spore inocula caused dilution of the initial *Ca*Gg bacterial population eventually leading to cured AM spores. *Ca*Gg was not essential to the survival or reproduction of the fungus. However, spores had a distinct phenotype in terms of the cytoplasm organization, vacuole morphology, cell wall organization, lipid bodies and pigment granules. The absence of bacteria severely affected presymbiotic fungal growth such as hyphal elongation and branching. However, at least under laboratory conditions, cured *G. margarita* formed mycorrhizal associations and also sporulated in the colonized roots (Lumini et al., 2007). Recent work supported the view that *Ca*Gg increases the environmental fitness and bioenergetics potential of *G. margarita* by priming mitochondrial metabolic pathways (Salvioli et al., 2016). The endobacterium influenced fungal growth, metabolism, and calcium signaling by targeting mitochondrial activity, upregulation of the genes involved in respiration, ATP production and reactive oxygen species (ROS) detoxification (Vannini et al., 2016). The authors concluded that - at least for the *G. margarita* BEG 34 isolate - the absence of endofungal bacteria causes delays in the growth of germinating mycelium, possibly affecting its overall ecological fitness.

Endofungal bacteria in EM symbiosis

In contrast to AM symbiosis, a relatively small number of plants live in symbiosis with ectomycorrhizal (EM) fungi, mainly forest trees in temperate and boreal ecosystems. EM symbioses are formed by a large number of fungal species, mainly Basidiomycetes, but also Ascomycetes. In the early 1990th the concept of mycorrhization helper bacteria (MHB) emerged to describe bacteria that help to establish a mycorrhizal symbiosis (Garbaye, 1994; Frey-Klett et al., 2007). This concept comprised all types of mycorrhiza and free as well as endocellular bacteria that support a symbiosis and thus appears somewhat indistinct. We focus here mainly on reports suggesting that EM also is associated with true endofungal bacteria. A rather specific though not endocellular association was described for the bacterium *Streptomyces* strain AcH 505 that improves mycelial growth and EM formation between the fly agaric fungus *(Amanita muscaria)* and spruce (*Picea abies*). Auxofuran was identified as the predominant growth promoting substance which was most effective at a concentration of 15 µM. Co-cultivation of the bacteria and *A. muscaria* stimulated auxofuran production in the fungus. Another example is the contribution of *Pseudomonas fluorescens* BBc6R8, isolated from a *Laccaria bicolor* sporocarp consistently promoting *L. bicolor*-Douglas fir (*Pseudotsuga menziesii*) EM formation. The prevalence of strain BBc6R8 in the soil was significantly enhanced by the presence of *L. bicolor* S238N in either the presence or absence of Douglas-fir roots, while in contrast its survival was not supported by non-mycorrhizal roots, suggesting that the strain *P. fluorescens* BBc6R8 depends more on the fungus than on the plant roots (Frey-Klett et al., 2007).

Bacterial proliferations also have recurrently been observed in fermentor cultures of the *L. bicolor* strain S238N, suggesting cryptic bacteria associated with this fungus. Endofungal bacteria were detected by Fluorescence in situ Hybridization (FISH) in pure fungal subcultures. They were small in size (0.5 µm in diameter), rare, and heterogeneously distributed in the mycelium. 16S rRNA gene sequence analysis identified those endobacteria as *Paenibacillus* spp. (Bertaux et al., 2003). However, *Paenibacillus* spp. have not been recovered from colonized plant samples, although pure fungal cultures served as inoculum (Bertaux et

al., 2005). Instead, samples taken from EM roots rather contained endofungal *Alphaproteobacteria*, which *vice versa* had not been detected in pure *L. bicolor* S238N cultures. Many *Alphaproteobacteria* also were detected outside the hyphae, in addition to bacteria belonging to other phyla, such as Actinomycetes and *Cytophaga-Flexibacter*. Thus, the authors speculated about an environmental origin of the endofungal *Alphaproteobacteria* (Bertaux et al., 2005).

Endofungal bacteria in the Sebacinalean symbiosis

The order Sebacinales is the most basal Basidiomycota group which contains fungi that undergo endophytic as well as mycorrhizal interactions with a broad spectrum of monocotyledonous and dicotyledonous plants found across all continents (Selosse et al., 2007; Weiss et al., 2011, Riess et al., 2014). The family *Serendipitaceae* comprises endophytes from the genus *Serendipita* such as *Piriformospora* (syn. *Serendipita*) *indica* that constitutes an excellent root endophyte model (Verma et al., 1998), especially due to available genome information, genetic tractability, and its broad host range that includes many important cereals and *Brassicaceae* such as the model plant *Arabidopsis thaliana* (for review Qiang et al., 2012). Unlike AM, *Serendipita* species are facultative biotrophic and thus can easily be cultured with synthetic medium in the absence of a plant (Deshmukh et al., 2006; Oelmüller et al., 2009). Endofungal bacteria identified in various *Serendipita* species belong to two genera of Gram-negative (*Rhizobium* and *Acinetobacter*) and two genera of Gram-positive (*Paenibacillus* and *Rhodococcus*) bacteria (Sharma et al., 2008; see figure 1). The most comprehensively studied example of a tripartite Sebacinalean symbiosis, including endofungal bacteria, is the association of *P. indica* with the Alphaproteobacterium *Rhizobium radiobacter* (*Rr*; syn. *Agrobacterium tumefaciens,* syn "*Agrobacterium fabrum*"; Figure 4). FISH using the *Rhizobium*-specific probe *Rhi-1247* confirmed the endocellular association of *Rr* with fungal spores and hyphae. Using quantitative PCR analysis, a ratio of 0.02 - 0.035 ng of bacterial DNA per 100 ng of *P. indica* DNA was determined (Sharma et al., 2008). This value is consistent with the low number of bacteria (2 - 20 per fungal cell) detected in *L. bicolor* (Bertaux et al., 2003; 2005). *R. radiobacter* was determined in the original strain of *P. indica*

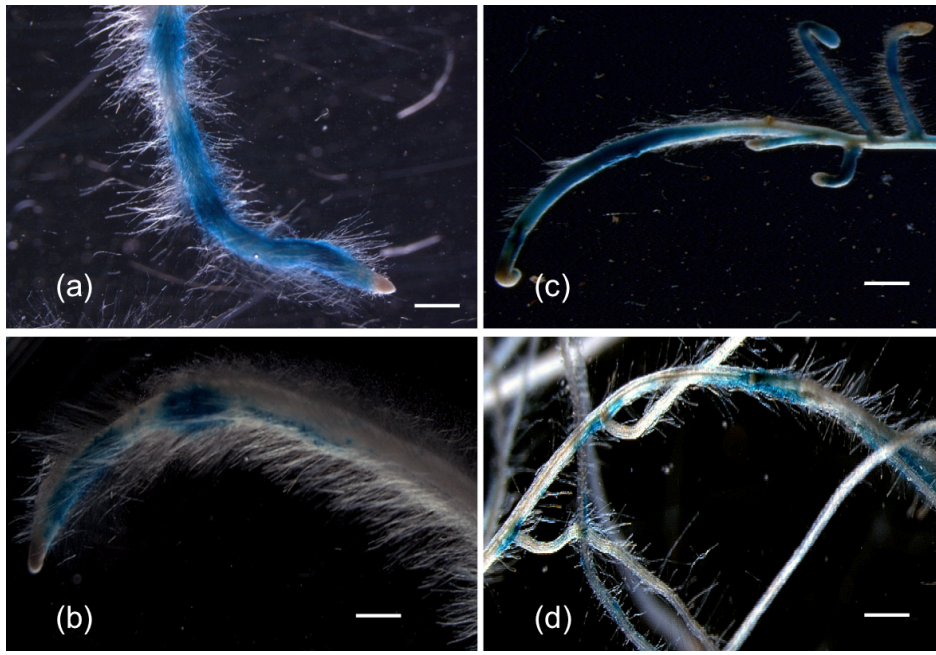


Figure 4. Colonization pattern upon inoculation of plant roots with the endofungal bacterium *R. radiobacter* F4 (*Rr*F4) that was isolated from a fungal *Piriformospora indica* culture. The endobacteria were labelled with the β-glucuronidase (GUS) reporter enzyme. Bacteria are visualized after X-Gluc treatment of roots at 7 (a,b) and 14 (c,d) days after dip inoculation. Staining of barley primary roots (a,b); lateral root protrusions and root hair zones of secondary roots of barley (c) and Arabidopsis (d). Scale bar = 2 mm.

DSM 11827 isolated from the Indian jar dessert and from three other *P. indica* cultures by the use of Denaturing gradient gel electrophoresis (DGGE) of 16S rRNA gene fragments amplified from fungal DNA extracts (Sharma et al., 2008). The strain *R. radiobacter* F4 (*Rr*F4) was isolated from *P. indica* DSM 11827 and propagated in axenic cultures with Luria-Bertani (LB) medium, showing that the bacterium is not entirely dependent on its fungal host. However, attempts to cure *P. indica* of *Rr*F4 have failed. Treatments with antibiotics such as spectinomycin and ciprofloxacin, which inhibit the growth of *Rr*F4 in pure cultures, merely transiently reduced the number of bacteria in the fungal mycelium as shown by the increase in the number of bacteria after prolonged cultivation in the absence of antibiotics. The fact that bacteria could not be killed inside the hyphae raised the speculation that *Rr*F4 enters an inactive state where it becomes insensitive to antibiotics. The protection against antibiotics may also be a survival strategy for bacteria in the rhizosphere which is commonly rich in antimicrobial compounds producing rhizobacteria. Nevertheless, a combination of protoplastation and antibiotics treatments reduced the abundance of endobacterial cells below the detection limit. Fungi regenerated from those protoplasts showed both reduced vegetative growth and chlamydospore formation suggesting a requirement of endobacteria for the fungal fitness (Guo et al., submitted). This situation is reminiscent of interactions of *Ca*Gg with G. *margarita* and *Burkholderia* spp. with *Rhizopus microspores*, where the absence of the respective bacterium also reduced fungal fitness (Lumini et al., 2007; Lackner et al., 2011).

The genome of *Rr*F4 is organized in a circular (2.8 Mb) and a linear chromosome (2.06 Mb), a tumor-inducing plasmid pTiF4 (0.21 Mb), and an accessory plasmid pAtF4 (0.54 Mb) and thus shows a high degree of similarity to the plant pathogenic *R. radiobacter* C58 (*Rr*C58; formerly: *Agrobacterium tumefaciens*; syn. "*A. fabrum"* C58; Goodner et al., 2001). The circular and linear chromosomes of *Rr*F4 had 100 and 80 singleton open reading frames (ORFs), respectively, that are not present in C58. Most of these ORFs were of unknown function and may be candidates for future studies to elucidate a potential role for the endofungal growth of *Rr*F4 and/or fitness of its fungal partner *P. indica* (Glaeser et al., 2016). Differences such as the loss of the T-DNA in the tumor-inducing (pTi) plasmids can explain the loss of *Rr*F4's pathogenicity (Glaeser et al., 2016). In contrast to obligate endofungal bacteria such as the non-cultivable *Ca*Gg, the genome of *Rr*F4 is not reduced, which is consistent with the situation in the cultivable endobacterium *Burkholderia rhizoxinica* (Lackner et al., 2011). Thus, the data are consistent with the hypothesis that *Rr*F4 forms a facultative symbiosis with *P. indica*, where the bacterium is still able to live independently outside its host. Supportive for this hypothesis, curing *Rr*C58 from its pTi plasmid resulted in a non-pathogenic strain with weak plant growth-stimulating activity in maize seedlings in non-sterile soil (Walker et al., 2013).

Endofungal bacteria enhance plant growth

The benefits or costs that fungal endophytes and endophyte-bacterial complexes extend to their plant hosts are greatly associated with plant health and seed production. Thus, it is important to address the effects endofungal bacteria have on both, disease resistance and yield. Fungi and bacteria can interact synergistically to stimulate plant growth through a range of mechanisms that include improved nutrient acquisition and inhibition of fungal pathogens (Artursson et al., 2006). In general, the beneficial potential of bacteria closely associated with the plant root can be inferred from the group of plant growth-promoting rhizobacteria (PGPR) that are in contact with the root surface, or rhizoplane, and increase plant yield by diverse mechanisms such as improved mineral uptake, disease suppression, or phytohormone production (Weller, 1988; Kloepperet al., 1991; Lugtenberg et al., 1991; Broek and Vanderleyden, 1995; Défago and Keel, 1995). Even so they are not "endofungal", PGPR have been reported to benefit associated fungi during interaction with their plant host. Certain groups of bacteria have been shown to accumulate to a higher extent in the mycorrhizosphere compared with other groups (Artursson et al., 2006 and references therein). PGPR also interact with AM fungi and have stimulatory impact on the growth of these fungi suggesting that they have at least an additional indirect effect on plant growth promotion (Garbaye, 1994). For example, association of *Pseudomonas putida* with AM fungi result in increased growth of clover plants, suggesting that PGPR may have properties that support both mycorrhizal establishment and function (Meyer and Linderman, 1986). Phylogenetically diverse endofungal bacteria detected in hyphae of diverse foliar Ascomycota endophytes of trees produced the plant-growth promoting hormone indole-3-acetic acid (IAA). Most of these bacteria were members of the Proteobacteria (Hoffman and Arnold, 2010). For instance, *in vitro* production of IAA by a fungal endophyte determined as *Pestalotiopsis* spp. (Pezizomycotina) that was isolated from foliage of a

coniferous host (*Platycladus orientalis*) was enhanced by the presence of *Luteibacter* sp. of the *Xanthomonadales*. IAA production by the endophyte-bacterial complex required L-tryptophan. The isolated and axenically cultured *Luteibacter* sp. did not produce IAA on a standard growth medium. However, culture filtrate from the endophyte-bacterium complex enhanced growth of tomato plants relative to filtrate from the endophyte alone. Given that the hormone produced by diverse plant-associated fungi can enhance plant growth and also suppresses, to a certain extent, plant defense such as the hypersensitive reaction (HR) and production of pathogenesis-related (PR) proteins, IAA production could be an important aspect of foliar endophyte-plant symbioses (Maor et al., 2004; Spaepen and Vanderleyden, 2011; Hoffman et al., 2013).

Inoculation of various plant species such as barley, wheat, and Arabidopsis with pure cultures of *Rhizobium radiobacter* F4 (*Rr*F4) promotes shoot and root growth, including lateral root branching (Sharma et al., 2008; Glaeser et al., 2016). These activities widely mimicked the effects *Rr*F4's host fungus *P. indica* has on plants. Yet, as the fungal host could not be completely cured from the endobacterium, it is still an unresolved question of whether beneficial plant biomass enhancement in the Sebacinalean symbiosis merely stems from *Rr*F4, the fungus *P. indica*, or the endophyte-bacterium complex (Guo et al., submitted).

In a forest nursery, the amount of phosphorus-solubilizing and siderophore-producing fluorescent pseudomonads was much higher in the Douglas-fir ectomycorrhizal fungus *L. bicolor* than in the surrounding root-free soil (Frey-Klett et al., 2005). The enhancement of the solubilization of rock phosphate occurred following formation of mixed biofilms between phosphate-solubilizing saprotrophic fungi and a *Bradyrhizobium elkanii* strain (Jayasinghearachchi and Seneviratne, 2005). The bacterial secondary metabolite responsible for plant growth promoting activity of *Amantia muscaria* through *Streptomyces* sp. AcH 505 is 5,6,7-trihydro-7-hydroxy-3-prolylbenzofuran-4-1, termed auxofuran, because of its auxin-reminiscent structure (Frey-Klett et al., 2007; Keller et al., 2006; Riedlinger et al., 2006). Upon treatment with auxofuran, expression of the *A. muscaria* gene acetoacetyl-CoA synthetase (*AmAacs*) was up-regulated, indicating activation of sterol biosynthesis (Riedlinger et al., 2006). Auxins and ethylene also have been implicated in inducing morphological changes in roots during mycorrhizal formation (Kaska et al., 1999), including the formation of lateral roots and dichotomous branching of short roots (Barker and Tagu, 2000).

Promotion of lateral root formation also is a commonly observed characteristic of MHB, essentially leading to an increase in potential contact points at which the plant and the ectomycorrhizal fungus can interact (Poole et al., 2001; Schrey et al., 2005). MHB can indirectly facilitate EM fungi's root colonization by inducing the release of signal molecules such as plant flavonoids (Frey-Klett et al., 2007) or suppressors of the plant's defence responses (Lehr et al., 2007). Moreover, root colonization by EM fungi may be facilitated be MHB's production of plant cell wall-digesting enzymes thereby enhancing penetration and spreading of the fungus within the root tissues (Mosse, 1962).

Acyl homoserine lactone production and quorum sensing of endofungal bacteria

Acyl homoserine lactones (AHLs) are well-known molecules produced by bacteria for their own communication termed quorum sensing (QS). More recently, AHLs were also implicated in beneficial activities bacteria have on plant yield and health (Schuhegger et al., 2006; Schikora et al., 2011; Schenk et al., 2014; Zarkani et al., 2013). Using a bacterial biosensor screening, AHL-producing endofungal bacteria were detected in antagonistic soil fungi (Kai et al., 2012). *In situ* detection by FISH analysis and electron microscopy confirmed the presence of endobacteria in the mycelium of the zygomycete fungus *Mortierella alpina* A-178 that produced the QS molecules. Release of these molecules by the fungus was subsequently proven by the detection of AHL from the supernatant of the liquid culture of the fungus. Amplification of 16S rRNA gene sequence fragments from fungal mycelium extracts indicated that a Beta-proteobacterium with 100% 16S rRNA gene sequence identity to *Castellaniella defragrans* (100 %) and a Gram-positive bacterium assigned to the genus *Cryobacterium* (99.8% 16S rRNA gene sequence identity) were present in the mycelium of *M. alpina* A-178. Antibiotic treatment enabled to cure the fungus, and the lack of AHL detection in the fungus confirmed that the AHL is produced by the endobacterium. Yet, how QS is involved in the interaction of fungus and bacterium needs to be elucidated by further studies. Similarly pure cultures of endofungal *Rr*F4, isolated from *P. indica*, also produce various classes of AHL that are known to

have growth promoting and induced resistance activity in plants (unpublished data). Significantly, mutants of *Rr*F4 that were compromised in AHL accumulation, showed reduced growth promoting activity when colonizing wheat or Arabidopsis plants. These merely preliminary findings suggest a more rigorous analysis of the role of AHLs in tripartite interactions. Noteworthy, *B. rhizoxinica*, lacks genes related to known QS systems (Lackner et al., 2011b).

Complexes of closely associated bacteria and beneficial fungi enhance local and systemic resistance to microbial pathogens

Fungus-bacterium complexes also contribute to plant protection against leaf and root pathogens. Various studies have demonstrated *in vitro* antagonistic activity exerted by mycorrhiza-associated bacteria on microbial plant pathogens (Schelkle and Peterson, 1996; Becker et al., 1999; Maier et al., 2004; Frey-Klett et al., 2007). The EM-associated *Pseudomonas fluorescens* strain BBc6R8 inhibited the growth of various root-pathogenic fungi belonging to the genera *Rhizoctonia*, *Fusarium*, *Phytophthora* and *Heterobasidion* in the Douglas-fir - *L. bicolor* interaction (Frey-Klett et al., 2005). In addition, a strain of *Paenibacillus* spp. isolated from the rhizosphere of sorghum proved to be compatible with AM development, but inhibited soil-borne fungal pathogens (Budi et al., 1999). This bacterial strain produced small peptides that were harmless to the symbiotic fungi, but were responsible for the antagonistic effects to pathogens (Selim et al., 2005). Cruz and Ishii (2008) isolated two bacteria from surface sterilized spores of *Gigaspora margarita.* Both isolates that were closest related to *Janthinobacterium* and *Paenibacillus* spp. inhibited growth of the soil-borne plant pathogens *Fusarium oxysporum* f. sp. *lactucae*, *Rhizoctonia solani*, and *Pythium ultimum* (Cruz and Ishii, 2008).

Pure cultures of the endofungal bacterium *Rr*F4 induced systemic resistance against various microbial leaf pathogens such as the powdery mildew fungus *Blumeria graminis* f.sp. *hordei* and the bacterium *Xanthomonas translucens* pv. *translucens* (*Xtt)*, when inoculated to barley or wheat roots, respectively. Similarly, *Rr*F4 also induced resistance in Arabidopsis to *Pseudomonas syringae* pv. *tomato* DC3000 (*Pst*) (Sharma et al., 2008; Glaeser et al., 2016). The underlying resistance mechanism in Arabidopsis depends on an operable jasmonate pathway, while mutants defective in genes governing SA accumulation and signaling where not compromised in *Rr*F4-mediated resistance (Glaeser et al., 2016).

Toxin-producing endofungal bacteria contribute to fungal virulence

The rice seedling blight fungus *Rhizopus microspores* (Mucoromycotina, Mucorales) severely affects rice fields in Asia that begins with an abnormal swelling of seedling roots and eventually results in death of the affected tissue or the complete plant. The fungus forms beneficial associations with the *Betaproteobacteria Burkholderia endofungorum* and *Burkholderia rhizoxinica* (Partida-Martinez et al., 2007b), which support the fungal host to parasitize plant tissue. *B. rhizoxinica* produces the phytotoxic polyketide metabolite rhizoxin (Partida-Martinez and Hertweck, 2005; Lackner et al., 2011) which blocks mitosis in eukaryotic cells, including plants, by binding to β-tubulin and thus serves the fungus as virulence factor (Sato *et al*. 1983). Due to an amino acid change in the tubulin protein, *Rhizopus* itself is tolerant to rhizoxin (Schmitt et al., 2008). Remarkably, rhizoxin had earlier been related to the fungal metabolism. However, treatment of the fungus with ciprofloxacin (40 µg ml^{-1}), an antibiotic active against bacteria but not against fungi, cured the fungus of the bacteria and concomitantly extirpated rhizoxin production (Partida-Martinez and Hertweck, 2005; Partida-Martinez et al., 2005). Consistent with this, *B. rhizoxinica* contains the gene cluster encoding rhizoxin biosynthesis (Partida-Martinez and Hertweck, 2007).

In contrast to the AM endofungal bacterium *Ca*Gg that resisted cultivation in cell-free medium and could only be investigated by using molecular methods, *B. rhizoxinica* could be isolated, grown in pure culture, and eventually reintroduced into a cured fungal host strain (Partida-Martinez and Hertweck, 2005; Scherlach et al., 2006; Partida-Martinez et al., 2007; Moebius et al., 2014). The bacterium is vertically transmitted through asexual reproduction units (vegetative spores) of its fungal host, a process that was discovered through re-infection of fungal mycelia using laser-mediated microinjection or co-cultivation with green fluorescent protein (GFP)-producing bacterial strains (Partida-Martinez et al., 2007a). Interestingly, in the absence of the endofungal bacteria, *R. microsporus* is compromised in asexual reproduction as evidences by a lack of mature sporangia and spores in mycelium of strains lacking bacteria. Formation of sporangia was restored upon reintroduction of bacteria, showing that reproduction

of the fungal host is strictly dependent on the intact association (Partida-Martinez et al., 2007a). Symbiont-dependent sporulation is a hallmark of close mutualistic relationships (Moran, 2006) and an elegant way to prevent formation of symbiont-free spores, thus securing the persistence of the symbiosis. Further experiment showed that the bacterial rhizoxin is not required for the bacterial infection process nor for fungal sporulation: Both the *B. rhizoxinica* wild-type strain and a mutant deficient in rhizoxin biosynthesis supported fungal sporulation in culture showing that other bacteria factors are necessary for the symbiosis. Supporting this notion, a Type III secretion system (T3SS) was discovered in *B. rhizoxinica* that controls a range of interactions between the bacterium and its fungal host, including invasion, intracellular transport of bacteria to hyphal tips and sporulation (Lackner et al., 2011). The entire gene cluster spans about 22,000 bp and contains 23 open reading frames (ORFs). In terms of primary sequence conservation and gene order, the gene cluster is similar to the hypersensitive response protein (hrp) locus coding for a T3SS that promotes virulence of the plant pathogen *Ralstonia solanacearum* (Cunnac et al., 2004). *B. rhizoxinica* T3SS$^-$ mutants defective in two T3SS genes, *DsctC* and *DsctT*, did not show growth defects or morphological phenotypes compared with the wild type when grown in culture. However, these mutants were strongly compromised in their fitness as only limited zones of infection were visible. As in cured fungal cultures, the fungal host infected with these mutants was affected in intracellular survival and failed to elicit formation of mature sporangia in an infection assay (Lackner et al., 2011).

Isolation of fungal endobacteria

Only few endofungal bacteria have been isolated from their host fungi. Cruz et al. (2008) described the isolation of endofungal bacteria from *Gigaspora margarita*. After bacteria were determined by PCR from surface sterilized spores, they were isolated by osmosis from fungal protoplasts. The protoplasts were generated from spores by using two enzymes, a lysing enzyme and catalase. Two oval cells forming bacteria were isolated by this cultivation approach, a Gram-negative bacterium closest related to *Janthinobacterium lividum* and a Gram-positive bacterium closest related to *Paracoccus polymyxa*. Both isolates showed antagonistic properties against the pathogenic fungi *Rosellinia necatrix*, *Pythium ultimum*, *Fusarium oxysporum* and *Rhizoctonia solani*.

The endobacterium *Rr*F4 was isolated in a similar manner from crushed mycelium of *P. indica* (Sharma et al., 2008), and the two endosymbiotic *Burkholderia* species, *B. rhizoxinica* and *B. endofungorum,* were isolated from the supernatant of disrupted mycelium of two strains of *R. microspores*. Whether the free living growth state of endobacteria occurs in nature as a part of the bacterial live cycle is unclear. Glaeser et al. (2016) speculated that the endobacterium of *P. indica* may be released by root colonizing fungi to penetrate into internal root tissue to enhance the biological activity. A clear proof by *in situ* detection is still missing.

Bacterial invasion into fungal cells

Although endofungal bacteria have been known for four decades, the mechanism by which they enter fungal cells is unresolved. A recent study showed that a type II secretion system (T2SS) of the endofungal bacteria *B. rhizoxinica* is required for the formation of the endosymbiosis (Moebius et al., 2014), based on genome mining for potential symbiosis factors and functional analyses. Comparative proteome analyses show that the bacterium releases chitinolytic enzymes (chitinase, chitosanase) and chitin-binding proteins. Genes encoding chitinase and chitosanase are highly expressed during the infection. The authors suggested that secreting these enzymes and presumably further effector proteins via a T2SS help to locally soften the fungal cell wall allowing bacterial entry and preventing the disintegration of fungal hyphae without permanent damage (Moebius et al., 2014). Consistent with this, a chitinase loss-of-function mutant lost its ability to enter fungal hyphae.

Conclusion

Elucidating the biology of tripartite associations between plants, higher fungi, and endofungal bacteria has shown that endofungal bacteria have various beneficial activities that support growth and development of their fungal hosts. The association of *Rhizopus microsporus* with *B. rhizoxinica* is an outstanding model, because the fungus can be cured from the endofungal bacteria and isolated bacteria can be grown in pure cultures, two prerequisites to fully elucidate the role of bacteria in fungi. That is why the role of endofungal bacteria in endo-, ecto-, and Sebacinalean symbioses is still hampered as the fungi either cannot by cured or endobacteria cannot be cultured outside the fungal mycelium. Accordingly, our understanding of these tripartite symbioses is far from being complete. New

strategies are needed to remove the bacteria from the fungal cytoplasm to enable comparisons of fungal effects on plants in the presence and absence of the bacterial symbionts. Despite these setbacks, it seems a more common phenomenon that root endophytic fungi host endobacteria that enhance the fungal fitness. In the cases the endofungal bacteria could be isolated and grown in pure culture they show similar biological activities than the fungal hosts itself. This makes it feasible that the fungus gains its full plant-colonizing activity from its intricate interaction with endobacteria.

Beneficial effect of pure endofungal bacteria also make them potentially interesting as application as biologicals that could further minimize the use of chemical fertilizers and increase the resistance of crop plants to a wider range of pathogens and pest, where the mechanism greatly relays on their direct antagonistic activity and their resistance-inducing potential. The present knowledge on endobacteria already shows the requirement towards unraveling a complex network of interphylum interactions, which is expected to have a previously unrecognized ecological impact.

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