Who lives in a fungus? The diversity, origins and functions of fungal endobacteria living in Mucoromycota

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Summary

Bacterial interactions with plants and animals have been examined for many years; differently, only with the new millennium the study of bacterial-fungal interactions blossomed, becoming a new field of microbiology with relevance to microbial ecology, human health, and biotechnology. Bacteria and fungi interact at different levels and bacterial endosymbionts, which dwell inside fungal cells, provide the most intimate example. Bacterial endosymbionts mostly occur in fungi of the phylum Mucoromycota and include Betaproteobacteria (*Burkhoderia*-related) and Mollicutes (*Mycoplasma*-related). Based on phylogenomics and estimations of divergence time, we hypothesized two different scenarios for the origin of these interactions (*early* vs *late bacterial invasion*). Sequencing of the genomes of fungal endobacteria revealed a significant reduction in genome size, particularly in endosymbionts of the Glomeromycotina, as expected by their uncultivability and host-dependency. Like endobacteria of insects, the endobacteria of fungi show a range of behaviours from mutualisms to antagonisms. Emerging results suggest that some benefits given by the endobacteria to their plant-associated fungal host may propagate to the interacting plant, giving rise to a three-level inter-domain interaction.

Introduction

In their introduction to a special issue of *Science* entitled "Manipulating the microbiota", Ash and Mueller (2016) wrote "*No man is an island*," quoting the poet John Donne. In recent years we have learned that animals and plants host thousands of microbes, many beneficial, some essential, and only a few deleterious. NGS approaches have enabled in-depth investigations of the microbial communities associated with animals and plants, asking "Who is there?", and "What are they doing?". Most surveys of animal- and plant-associated microbes so far have focused on bacteria and have demonstrated that they mainly participate in immune regulation and barrier defense (Haney and Ausubel, 2015). Conversely, fungi have been mostly neglected, even though recent studies demonstrated the unique biological and ecological role of fungal communities (Shakya *et al*., 2013; Coleman-Derr *et al*., 2016).

Since the discovery of antibiotics, fungi and bacteria have been assumed to interact antagonistically, but new ideas have emerged in microbial studies: as elements of the same microbiota, fungi and bacteria may interact non-antagonistically (Olsson *et al*., 2017). Indeed, emerging work has discovered an increasing number of cooperative bacterial-fungal associations, giving rise to a new field of microbiology (Frey-Klett *et al*., 2011). These inter-domain interactions occur in different ways: bacteria may loosely associate with the hyphal surface, or may show some partner specificity, indicating potential metabolic complementation (Schroeckh *et al*., 2009; Olsson *et al*., 2017). The most intimate interaction takes place when bacteria live inside the fungal cells as endobacteria. Irrespective of their genetic and functional diversity, fungus-associated bacterial communities constitute a novel type of microbiota, the fungal microbiota (Desirò *et al*., 2014).

This mini-review summarizes our current knowledge on the endobacteria of fungi, with particular attention to the Glomeromycotina (arbuscular mycorrhizal fungi,

AMF), and uses these AMF as a paradigm to better understand the diversity, origins, and functions of fungal endobacteria.

The places they call home: Distribution and diversity of fungal endobacteria

In 1970 Barbara Mosse reported the presence of bacteria-like organisms in the cytoplasm of Glomeromycotina spores. In the words of Neil Armstrong, who landed in the moon the year before, we could say: "*One small step for a woman, one giant leap for science*". Indeed, Mosse's finding offered the first glimpse of this intimate, inter-domain interaction. In later decades, before the invention of PCR, many pioneering studies provided additional morphological evidence of the presence of these bacteria-like organisms in *Endogone flammicorona* (Bonfante and Scannerini, 1976; 1977) and several AMF species (references in Scannerini and Bonfante, 1991), demonstrating that endobacteria can be found widely in fungi. With the new millennium, the study of bacterial-fungal interactions (BFI) blossomed, enjoying a boost from the suitable-for-all availability of molecular biology techniques.

Numerous recent studies have identified endobacteria in several fungal lineages (Olsson *et al*., 2017). Ascomycota and Basidiomycota were shown to harbour Gammaproteobacteria (Arendt *et al*., 2016) and Alphaproteobacteria (Sharma *et al*., 2008; Glaeser *et al*., 2016), respectively. However, most of the fungal endobacteria hitherto described were identified in members of the phylum Mucoromycota (Spatafora *et al*., 2016). The endobacteria of the Mucoromycota mainly involve Betaproteobacteria (*Burkhoderia*-related) and Mollicutes (*Mycoplasma*-related). One exception is the cyanobacterium *Nostoc puntiforme* that resides within *Geosiphon pyriformis* (Schüßler and Kluge, 2001).

One of the better-known example involving a betaproteobacterium is the rice pathogenic fungus *Rhizopus microsporus* (Mucoromycotina), the causal agent of rice seedling blight, whose pathogenicity is due to the presence of the endosymbiont

Burkholderia rhizoxinica (Partida-Martinez and Hertweck, 2005). Indeed, *B. rhizoxinica* produces the rhizoxin used as a virulence factor by the fungus (Partida-Martinez and Hertweck, 2007). Interestingly, this facultative endobacterium affects the vegetative reproduction of the fungal host, controlling the formation of sporangia and spores (Partida-Martinez *et al*., 2007). Much recent attention has been devoted to the interaction between a *Burkholderia*-related endobacterium (BRE) and several strains of *Mortierella elongata* (Mortierellomycotina) (Sato *et al*., 2010; Ohshima *et al*., 2016; Uehling *et al*., 2017). This obligate endosymbiont was recently named *Mycoavidus cysteinexigens*, since it requires cysteine to grow without the fungal host (Ohshima *et al*., 2016). Interestingly, recent studies (Li *et al*., 2017; Uehling *et al*., 2017) demonstrated that the presence of these endobacteria can strongly affect the metabolism of *M. elongata*: the wild-type strain with endobacteria showed a lower growth rate, compared to the strain that was cured (*i.e.* devoid of endobacteria), suggesting that the fungus experiences a metabolic cost for accommodating *Mycoavidus*.

Also fungi of the Glomeromycotina interact with Betaproteobacteria. An obligate rod-shaped BRE named *Candidatus* Glomeribacter gigasporarum (*Ca*Gg) (Bianciotto *et al*., 2003), was detected in several species of the family Gigasporaceae (Mondo *et al*., 2012; Desirò *et al*., 2014). Its phylogenetically closest relative turned out to be *Mycoavidus* (Ohshima *et al*., 2016; Uehling *et al*., 2017). Like *Mycoavidus* (Sato *et al*., 2010), diverse genetic variants of *Ca*Gg were identified, thus casting doubt on the existence of a unique *Ca*Gg species (Desirò *et al*., 2014). Notwithstanding the different *Ca*Gg phylotypes detected, each fungal host harbours a genetically uniform *Ca*Gg population (Mondo *et al*., 2012; Desirò *et al*., 2014). Interestingly, the same genetic uniformity was observed in the Betaproteobacteria populations found in *Rhizopus* (Lackner *et al*., 2011) and *Mortierella* (Sato *et al*., 2010).

A second type of endosymbiont resides within the spores and hyphae of the Glomeromycotina, and this coccoid endobacterium represents a taxon of Mollicutes/*Mycoplasma*-related endobacteria (MRE) (Naumann *et al*., 2010; Desirò

et al., 2013; Desirò *et al*., 2014; Toomer *et al*., 2015). This novel bacterial taxon, whose biology is still little known, has been recently accomodated in the novel genus Candidatus *Moeniiplasma* and named *Candidatus* Moeniiplasma glomeromycotorum (*Ca*Mg) (Naito *et al*., 2017). Like *Ca*Gg, *Ca*Mg does not appear to be able to grow outside of their fungal hosts, a feature that places it among the obligate endosymbionts. *Ca*Mg occurs widely across the Glomeromycotina and, unlike BRE, multiple *Ca*Mg populations can inhabit a single fungal strain: up to three highly dissimilar 16S rDNA *Ca*Mg phylotypes were identified in a single spore (Naumann *et al*., 2010; Desirò *et al*., 2014; Toomer *et al*., 2015). Notwithstanding the remarkable level of diversity, all the *Ca*Mg 16S rDNA sequences retrieved from AMF cluster within a monophyletic clade, sister to the Mycoplasmatales and Entomoplasmatales. Regardless of the internal phylogenetic structure, the more accurate multigene phylogenetic reconstructions placed this motley lineage of Mollicutes within the Mycoplasmataceae, close to the *Mycoplasma* species (Naito *et al*., 2015; Torres-Cortés, *et al*., 2015). The impressive diversity that characterizes these enigmatic microbes seems to be determined by several factors such as ultrarapid mutation rate, vertical transmission, activity of mobile genetic elements, active recombination machinery and apparent retention of the ability to conduct horizontal gene transfer (Naito *et* al., 2015; Toomer *et al*., 2015; Naito and Pawlowska, 2016a; 2016b).

The Glomeromycotina are not the only fungi that harbour MRE, since *Ca*Mg-related endobacteria were identified in fruiting bodies of several strains of *Endogone* (Mucoromycotina) (Desirò *et al*., 2015a) and recent results reported their presence in *Sphaerocreas pubescens* (Mucoromycotina) (Desirò *et al*., 2015b; Takashima *et al*., 2015). Like the AMF-associated MRE, multiple and highly dissimilar populations can co-exist in the same Mucoromycotina fungal strain. Despite their striking diversity (up to 21.8% sequence divergence with AMF-associated MRE phylotypes), the 16S rDNA phylogeny placed *Endogone*-associated MRE with those living in the Glomeromycotina. Interestingly, MRE from *Endogone* cluster within a new

phylogenetic group, clearly distinguishable from the AMF-associated MRE groups (Desirò *et al*., 2015a). The finding of a novel group of MRE in another fungal lineage, the Mucoromycotina, suggests that these endosymbionts are likely more widespread than expected. It would therefore be extremely interesting to know whether the third Mucoromycota subphylum, the Mortierellomycotina, host MRE.

Conjectures about the origin of fungal endosymbiosis

The presence of phylogenetically related endosymbionts in different fungal hosts offers insights about the origin and evolution of these inter-domain interactions. The *Ca*Gg symbiosis appears to have existed for at least 400 MY (Mondo *et al*., 2012), beginning before the diversification of the Gigasporaceae (VanKuren *et al*., 2013), and suggesting a scenario where a BRE was already associated with the common ancestor of the Gigasporaceae. Similarly, the *Mycoavidus-Mortierella* association seems to have originated in the mid-Paleozoic, most likely over 350 MYA (Uehling *et al*., 2017). Further phylogenomic analyses and divergence time estimations placed the separation of the *Ca*Gg and *Mycoavidus* lineage from the free-living *Burkholderia* some 350 MYA, while their fungal hosts (Glomeromycotina and Mortierellomycotina, respectively) diverged at 358-508 MYA (Uehling et al., 2017). On the one hand, *Ca*Gg and *Mycoavidus* seem to have diverged from a common ancestor; on the other hand, *B. rhizoxinica* seems to have had an independent origin, sharing with the free-living *Burkholderia* its most recent common ancestor. Therefore, it could be hypothesized that a free-living *Burkholderia*-related bacterium (BRB) diversified into two ancestral lineages, which evolved as i) mammalian and plant pathogens, saprotrophic species, plant-associated microbes (see references in Estrada-de los Santos *et al*., 2013), and facultative endosymbionts of fungi (*B. rhizoxinica*) or ii) obligate endobacteria that mostly lost their capacity to grow outside of their fungal hosts (*Ca*Gg and *Mycoavidus*). Accordingly, it is fascinating to hypothesize that the common ancestor of *Ca*Gg and *Mycoavidus* was already

dwelling within the mycelium of the ancestral fungal lineage that later produced the Glomeromycotina and Mortierellomycotina, thereby implying an *early bacterial invasion* of the fungal host (Figure 1). However, the rather limited distribution of these endosymbionts could undermine the *early bacterial invasion*. Indeed, we would expect the broad presence of BRE in most of the fungal species that originated following the *invasion*. The presence of *Mycoavidus* exclusively within *M. elongata* may be attributed to limited investigation of the Mortierellomycotina; however, the Gigasporaceae seem to represent the only "fungal environment" where *Ca*Gg can be retrieved. The absence of *Ca*Gg in most of the Glomeromycota lineages might be the result of *secondary losses* of the bacterial partner. Alternatively, it might suggest a different scenario entailing a *late bacterial invasion*, which may have occurred when the evolutionary lines leading to the Glomeromycotina and Mortierellomycotina had already separated (Figure 1).

The mechanisms of *invasion* also remain unknown. AMF hyphae can be damaged by other fungi (Lace *et al*., 2015) or by grazing soil fauna (Hedlund *et al*., 1991; Gange, 2000). This could have allowed an ancestral free-living BRB to invade the fungus through breaks in its wall. At the same time, we cannot exclude a more direct role of the bacterium in breaking the fungal wall and colonizing the fungus: indeed, there are several examples of Betaproteobacteria with chitinolitic capacities (Shimosaka *et al*., 2001; Shu-Chang *et al*., 2004). Further, a crucial role in the *invasion* process of *Rhizopus* was attributed to the *B. rhizoxinica* T2SS, which releases chitinolytic enzymes (Moebius *et al*., 2014). Similarly, *Ca*Gg possesses T2SS and T3SS, which are differentially expressed through the fungal life cycle (Ghignone *et al*., 2012). However, bacterial chitinases were not detected in the currently available *Ca*Gg genome, suggesting that nowadays *Ca*Gg could not be able to invade a fungus using a similar mechanism.

We can make similar conjectures about the origin and evolution of MRE. The widespread distribution of these endobacteria in the Glomeromycotina suggested that the MRE *invasion* occurred before the Glomeroycotina radiation, more than 400

MYA (Naumann *et al*., 2010). The finding that *Endogone* harbors MRE pushed Desirò and colleagues (2015) to hypothesize that this inter-domain interaction was even older and originated before the split between the Glomeromycotina and Mucoromycotina. This scenario assumes the existence of an ancestral MRE in the common ancestor of these two fungal lineages, thereby implying an *early bacterial invasion* of the fungal host. However, this scenario can be undermined by divergence time estimations. The divergence of the Mycoplasmataceae was estimated at 410 MYA (Maniloff, 2002), whereas the Mucoromycotina are believed to have split from the ancestral Glomeromycotina-Mortierellomycotina lineage some 500-600 MYA (Chang *et al*., 2015; Uehling *et al*., 2017), before the appearance of MRE. These results support the alternative scenario of a *late bacterial invasion*, which may have occurred when the evolutionary lines leading to the Glomeromycotina, Mortierellomycotina and Mucoromycotina were already separated (Figure 1).

Naito and colleagues (2015) suggested that the origin of MRE resulted from a host-switching event from animals to fungi by an ancestral MRE (Naito *et al*., 2015). Other lineages of Mollicutes, such as *Entomoplasma*, *Mesoplasma*, and *Spiroplasma* can be associated with animals, such as arthropods (Tully *et al*., 1993), which, in turn, are known to graze on fungal hyphae (Hedlund *et al*., 1991; Gange, 2000). Thus, in support of the host-switching hypothesis (Naito *et al*., 2015), it is intriguing to suppose that ancestral soil invertebrates, already associated with the MRE ancestor, may have acted as vectors, allowing the *invasion* of fungi. This novel scenario provides an interesting parallel with *Phytoplasma*, another lineage of uncultivable Mollicutes, which are insect-transmitted pathogenic agents of numerous plant species (Weintraub and Beanland, 2006). However, assuming the existence of an ancestral free-living Mollicutes/*Mycoplasma*-related bacterium (MRB), it also needs to take into consideration the possibility that the colonization of fungal hosts may have occured directly from the soil, when the mycelium was damaged by other fungi or soil invertebrates.

Regardless of the nature of the symbiosis, and how and when bacteria settled within fungi, these inter-domain associations may be included among the oldest interactions on Earth. Furthermore, these interactions all involve plant-associated fungi, therefore raising questions about the influence of endobacteria on the history and evolution of plant-fungal symbiosis. It is intriguing to hypothesize that endobacteria may have had a role, as their fungal hosts (Field *et al*., 2015), during one of the major turning points in evolution of the planet, the conquest of land by plants.

Keeping the home fires burning: Analysis of endobacterial genomes to understand endobacterial functions

One common feature of the endobacteria so far identified in AMF is their vertical transmission, a modality used by these endosymbionts, which obligately depend on their host, to move from one fungal generation to the next. By contrast, the relationship is facultative for the fungus; for example, *Gigaspora margarita* can proliferate in the absence of *Ca*Gg (Lumini *et al*., 2007) and several other AMF strains devoid of *Ca*Mg can be propagated under laboratory conditions (Naumann *et al*., 2010). Vertical transmission and obligate dependence on the host may imply that endobacteria complement their metabolism using metabolites from their partner. The genome sequencing of both *Ca*Gg (Ghignone *et al*., 2012) and *Ca*Mg (Naito *et al*., 2015; Torres-Cortés *et al*., 2015) has largely confirmed the fungal-host dependency hypothesis. The size of the *Ca*Gg genome (1.7- 1.9 Mb) and the more strongly reduced genomes of *Ca*Mg (0.7 to 1.3 Mb) are consistent with their uncultivable status.

Indeed, they have a smaller genome when compared to *B. rhizoxinica* (3.7 Mb) (Lackner *et al*., 2011) and *M. cysteinexigens* (~2.6 Mb) (Fujimura *et al*., 2014; Uehling *et al*., 2017) that can grow independently, outside of their fungal hosts. The possibility to maintain *B. rhizoxinica* in pure culture allowed Moebius and colleagues (2014) to provide experimental evidences on the mechanisms underlying the

colonization process of *Rhizopus*.

AMF endobacteria do not possess metabolic pathways producing essential amino acids. For example, *Ca*Gg lacks the ability to biosynthesize arginine, isoleucine, leucine, methionine, phenylalanine, tryptophan, histidine, and valine, while *Ca*Mg has even more strongly reduced capacities. At the same time, *Ca*Gg contains the full operon for vitamine B12 synthesis, and is equipped with many amino acid permeases and transporters (Ghignone *et al*., 2012). By contrast, only a few putative nutrient transporters were annotated in *Ca*Mg genomes, such as a putative arginine-ornithine antiporter (Naito *et al*., 2015; Torres-Cortés *et al*., 2015).

Interestingly, when a hierarchical clustering analysis of KEGG metabolic pathways was applied to *Ca*Gg and 28 other bacterial genomes, the genomic features grouped *Ca*Gg together with insect endosymbionts, including *Baumannia cicadellinicola* and *Wolbachia* spp. (Ghignone *et al*., 2012). The same analysis applied to *Ca*Mg placed it with obligate endosymbionts of insects with reduced metabolic capacities (Torres-Cortés *et al*., 2015), including *Ca*. Carsonella ruddii and *Ca*. Sulcia muelleri, which possess some of the smallest characterized bacterial genomes (McCutcheon and Moran, 2012). These findings indicate that the genomes of phylogenetically distinct lineages of endobacteria have been similarly shaped by the selection pressure generated within diverse eukaryotic hosts, providing evidence of convergent evolutionary adaptation to an intracellular lifestyle (Ghignone *et al*., 2012). Curiously, *B. cicadellinicola* and *Ca.* Sulcia muelleri may co-reside in the same host, *Homalodisca coagulata*, where they reveal functional complementation (Cottret *et al*., 2010). Similarly, *CaGg* and *Ca*Mg have been found to coexist within the same fungal strain (Desirò *et al*., 2014). Thus, further work is required to address whether functional complementation also occurs between the two endosymbionts when they dwell together inside the same fungal niche. Moreover, the identification of mobile genetic elements in *Ca*Gg (Ghignone *et al*., 2012) and *Ca*Mg (Naito and Pawlowska, 2016b) offered new insights into the possibility of gene exchange between the two endobacterial lineages.

While the genome analysis of AMF endobacteria has nicely revealed the mechanistic basis of their dependency on the host, the reasons why many fungal strains have maintained their bacterial guests for hundreds of million years have remained unknown. Using a stable endosymbiont-free strain of *Gigaspora margarita* (B-), multiple morphological and "omics" approaches have been applied to directly compare the B- line with the wild-type strain hosting the endobacterium (B+). Interestingly, in spite of its success in colonizing the plant host, the B- line is impaired in the mycelial growth, has a different spore wall structure, and sometimes produces fewer spores than the B+ line (Lumini *et al*., 2007). Consistent with these features, transcriptome analysis showed that *Ca*Gg has a stronger effect on the pre-symbiotic than the symbiotic phase of the fungal host (Salvioli *et al*., 2016). The coupling of transcriptomics and proteomics with physiological and cell biological approaches demonstrated that *Ca*Gg raises the bioenergetic capacity of the fungus, increasing its ATP production and respiration, and eliciting mechanisms to detoxify ROS (Salvioli *et al*., 2016; Vannini *et al*., 2016). In this scenario, many proteins specifically involved in endogenous ROS detoxification were found to be upregulated in the B+ line, which indeed produced more H_2O_2, but also had higher antioxidant capacities (Vannini *et al*., 2016). The fungal mitochondrion and its main metabolic pathways (ATP synthesis, respiration, ROS metabolism) appear therefore to be particularly sensitive to the presence of the bacteria.

Much experimental evidence has offered insights into the complex molecular events that directed the evolution of endosymbionts into contemporary organelles (Dyall *et al*., 2004). In line with these observations, and acknowledging that the distinction between endosymbiont and organelle is not always clear-cut, all the data generated so far might suggest a scenario where *Ca*Gg seems to act as a "mitochondrion-like organelle". In addition, the antioxidant activities elicited in the fungus by *Ca*Gg were also observed in the plant when colonized by the B+ strain (Vannini *et al*., 2016). Thus, even if the B- line is able to maintain its mycorrhizal capacities, it might be hypothesized that the ecological functionality of the AM symbiosis is positively

affected by the presence of *Ca*Gg (Figure 2). This hypothesis provides a further element to the idea that, like their animal counterparts, plants are never alone!

Home Sweet Home: Conclusions

In the past few years, the scientific community has made giant strides in the study of BFI. Today, this new field of microbiology has assumed substantial relevance not only to microbial ecology, but also human health and biotechnology (Frey-Klett *et al*., 2011; Netzker *et al*., 2015). In this changing context, interest in the endobacteria of fungi, which have long been considered an oddity, has blossomed. Indeed, the combination of different "omics" approaches is revealing an unexpectedly widespread distribution and is allowing us to gradually understand the biological functions and evolution of these microbes.

Most of the described examples of endobacterial-fungal interactions involve fungi in the Mucoromycota. These fungi possess a coenocytic mycelium (*i.e.* they lack or have few transverse septa), a feature that endobacteria might prefer. Indeed, the absence of physical barriers could facilitate bacterial movement along the hyphal network (Desirò *et al*., 2014; 2015a). Thus, we wonder whether the Mucoromycota, with their coenocytic mycelium, represent the more suitable niche where endobacteria can thrive, or if endobacteria could also inhabit other groups of fungi, such as the Dikarya. From the bacterial side, and irrespective of the host range, the diversity of fungal endobacteria discovered so far is mostly limited to two bacterial lineages, *Burkholderia* and *Mycoplasma*. Are they the only ones? Or do fungal endobacteria cover a broader taxonomic range, as for endobacteria-insect associations (Moran, 2001; Wernegreen, 2002)?

The rather limited diversity and distribution of fungal endobacteria cannot yet be compared to the one of insect endosymbionts; however, their biological roles offer interesting parallels. Insect endosymbioses show a continuity of behaviours from true

symbionts, through weak pathogens, to sex manipulators (Moran *et al*., 2008; McLean *et al*., 2016). Fungal endosymbioses behave likewise: *B. rhizoxinica* seems to be a true endosymbiont, with a positive effect on *Rhizopus*, similar to *Ca*Gg, which has a positive effect on *G. margarita*. By contrast, based on predictions of evolutionary theory, Toomer and colleagues (2015) suggested that *Ca*Mg may behave as antagonist of AMF. Lastly, even if it cannot be strictly defined as a sex manipulator, *B. rhizoxinica* affects the vegetative reproduction of its fungal partner (Partida-Martinez *et al*., 2007).

Similar to some obligate insect endosymbionts, such as *Buchnera*, endobacteria of fungi sequenced so far (particularly the AMF endobacteria) have revealed relevant genome reductions that entail host-dependency and, as a consequence, a range of difficulties in growing these bacteria in pure culture. As for insects, the interaction is facultative for the fungus and, moreover, has only been maintained in some lineages, suggesting that endobacteria are not essential for the evolutionary success of the hosts. Again this is true for endobacteria-insect interactions. For example, *Wolbachia* is one of the most widespread endobacteria, being present in around 40% of arthropod species. However, within a given species, usually *Wolbachia* infects most or only a few individuals (Zug and Hammerstein, 2012). For example, *Aedes aegypti* may host or not host such bacteria, and exciting research has demonstrated that when *Wolbachia* is introduced into the mosquitoes that lacked bacteria, this can stop the transmission of dangerous viruses that grow inside the mosquito and are transmitted to people (Hoffmann *et al*., 2011).

Compared to these mature fields, studies of endobacterial-fungal interactions are in their infancy. However, it is worthwhile to note that insects and fungi share common metabolic pathways, such as chitin biosynthesis, suggesting that the shift from a free-living lifestyle to obligate mutualism inside eukaryotic cells overcame similar structural barriers. Thus, we can ask whether the endobacteria of insects, which made an evolutionary transition from a free-living lifestyle to obligate mutualism (Hosokawa *et al*., 2016), faced similar challenges.

Focusing on endobacterial-fugal interactions, we can draw a portrait of an ancient scenario where soil is the main character, operating as a microbial reservoir where myriad organisms thrive together. Thus, it may be hypothesized that soil, with its living components, has acted as a facilitator in transferring free-living bacteria inside fungi. This has offered important opportunities for horizontal gene transfer in both directions, contributing to shaping the fungal genomes. The taking home-message is that fungal endobacteria are probably active participants, rather than silent occupants of their fungal homes.

Conflict of Interest

The authors declare no conflict of interest.

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Figure captions

Figure 1 Schematic representation of the two hypothetical scenarios of *bacterial invasion* (*early* vs *late*) at the basis of the origin of the association between BRE (in red) and MRE (in blue), and members of the Mucoromycota. **BRE**: The *early bacterial invasion* (left side) assumes the existence of an ancestral free-living BRB that invaded the common ancestor of the Glomeromycotina and Mortierellomycotina. During the separation of the fungal hosts, BRE have been diversified into *Candidatus* Glomeribacter gigasporarum and *Mycoavidus cysteinexigens*, and maintained only in

the Gigasporaceae (a family in the Diversisporales) and *Mortierella*, respectively. On the contrary, the *late bacterial invasion* (right side) entails a subsequent *invasion* by an ancestral free-living BRB, which may have occurred when the evolutionary lines leading to the Glomeromycotina and Mortierellomycotina had already separated, but before the diversification of the Gigasporaceae. Differently, *Burkholderia rhizoxinica* has had an independent origin, sharing with the free-living *Burkholderia* its most recent common ancestor. Irrespective of the *bacterial invasion* scenario, *Ca*Gg is absent in some Gigasporaceae strains and most of the Glomeromycotina and Mortierellomycotina lineages, and that might be the result of *secondary losses* of the endobacterial partner. **MRE**: The *early bacterial invasion* (left side) assumes the existence of an ancestral free-living MRB that invaded the ancestor of the Mucoromycota, and began its evolutionary path toward obligate mutualism. During the diversification of the Mucoromycota, MRE have been maintained in the Glomeromycotina and Mucoromycotina, whereas it is still unknown whether the Mortierellomycotina maintained these coccoid endosymbionts. By contrast, the *late bacterial invasion* (right side) entails a subsequent *invasion* by an ancestral free-living MRB, which may have occurred when the evolutionary lines leading to the three Mucoromycota subphyla had already separated. However, regardless of the *bacterial invasion* scenario, MRE are absent in several fungal lineages or strains and that might be the result of *secondary losses* of the endobacterial partner. Legend: *Burkholderia rhizoxinica* (*Br*); *Candidatus* Glomeribacter gigasporarum (*Ca*Gg); *Mycoavidus cysteinexigens* (*Mc*); free-living BRB (FLB); free-living/animal-associated MRB (FLM); bacterial invasion event (arrow); presence of bacteria/endobacteria (thick line); absence of endobacteria in at least one fungal lineage/strain (thin line); absence of endobacteria in at least one fungal lineage (species, genus, family or order) (thin line with a cross); unknown/there are no data available about the presence/absence of endobacteria (double thin line with a question mark).

Figure 2

Schematic comparison of the colonization success of *Gigaspora margarita* with (B+) and without (B-) its endosymbiont (*Ca*Gg). When compare to the B+ strain, the growth of the germinating mycelium from a B- spore is slower and, when the host plant root is relatively distant (~10 cm from the spore), stops after reaching a few centimeters (5 cm) (Lumini *et al*., 2007). Further, the B- strain often produces a lower number of spores than the B+ strain (Salvioli *et al*., 2016). Thus, in the words of Charles Darwin "*survival of the fittest*", these differences make the B+ strain the fittest one. AMF are obligate biotrophs, that is, they need a plant host to complete their life cycle. In natural conditions, the capacity to grow faster and for a longer distance/time may provide the B+ strain a greater chance of success in finding and reaching a host plant root, and then reproducing. On the contrary, the B- strain may have more difficulties in contacting plant host roots and, accordingly, completing its life cycle (grey spores). As a consequence, over the generations (from left to right), it might occur a decrease of the B- lines and a predominance of B+ lines in the soil.

Abbreviation list

AMF: arbuscular mycorrhizal fungi

BFI: bacterial-fungal interactions

BRB: *Burkholderia*-related bacterium

BRE: Burkholderia-related endobacterium

*Ca*Gg: *Candidatus* Glomeribacter gigasporarum

*Ca*Mg: *Candidatus* Moeniiplasma glomeromycotorum

MRB: Mollicutes/*Mycoplasma*-related bacterium

MRE: Mollicutes/*Mycoplasma*-related endobacteria

NGS: Next generation sequencing

ROS: Reactive oxygen species

T2SS: Type II secretion system