Chapter 39 Ecology and Evolution of Fungal-Bacterial Interactions

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Ecology and Evolution of Fungal-Bacterial Interactions

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I. General Introduction

The propensity of fungi to synthesize compounds active against bacteria (Broadbent 1966) and the predilection of bacteria to produce antifungals (Kerr 1999) gave rise to a paradigm that interactions between representatives of these two groups of organisms are of an antagonistic nature. While, indeed, evidence for fungal-bacterial antagonisms is abundant (Espuny Tomas et al. 1982; Leveau and Preston 2008; Susi et al. 2011; Palaniyandi et al. 2013; Pawlowska et al. 2012; Pliego et al. 2011), the recent accumulation of newly discovered associations in which fungi cooperate with bacteria (Kobayashi and Crouch 2009; Frey-Klett et al. 2011) indicates that such reciprocally beneficial interactions are more common than previously thought. As functional and mechanistic aspects of many of these interdomain relationships were reviewed in detail elsewhere (Grube and Berg 2009; Kobayashi and Crouch 2009; Peleg, Hogan, and Mylonakis 2010; Frey-Klett et al. 2011; Martin and Schwab 2012; Scherlach, Graupner, and Hertweck 2013), our discussion will focus on factors that contribute to their stability over ecological and evolutionary time. We hope that, by directing attention to this important but currently neglected aspect of fungal-bacterial interactions, we will inspire new directions of research on the biology of these organisms.

II. Definitions and Concepts

We use the term **symbiosis** in the de Bary's sense of "the living together of unlike organisms", without implications whether this relationship has positive or negative fitness consequences for any of the interacting partners (Martin and Schwab 2012). Thus in terms of fitness outcomes, the symbiosis can assume the forms of a **mutualism** (+/+), **commensalism** (+/0), and **antagonism**,

including **competition** (–/–), **amensalism** (–/0), **parasitism** and **predation/grazing** (–/+) (Lewis 1985). We doubt that strictly **neutral relationships** (0/0) exist among the symbiotic partners. We recognize that practically all biota on the planet are components of stabile assemblages of organisms, referred to as **metaorganisms** (Bosch and McFall-Ngai 2011). Although not ideal, this term is reasonably well defined and increasingly coming into use (Trinchieri 2014; Biagi et al. 2012). We employ it in our discussions of entities formed in the process and as a consequence of fungal-bacterial interactions (Fig. 1). Thus it is the metaorganism that survives in nature and changes over time due to evolution of its individual constituents, their composition, and the roles in the metaorganism. It is important to note that fungal-bacterial metaorganisms may be, in turn, components of higher-level metaorganisms comprising also plant or animal hosts. We refer to the fungal constituents of the fungal-bacterial metaorganism as the **hosts** and the bacterial partners as the **symbionts**. Both hosts and symbionts can be represented by a single species, or they can each comprise a multi-species consortium in which different species interact with each other. In terms of physical interface between the partners, bacterial symbionts can act as **endobionts/endosymbionts** living intracellularly inside the hyphae, or as **ectobionts/epibionts/ectosymbionts/episymbionts** associated with the surface of the hyphae or in the close vicinity of the hyphae, often in biofilms consisting of several layers of bacteria held together by a matrix. Metaorganism formation can take several routes. Most known associations of fungi with bacteria are **non-heritable,** with bacterial symbionts assembled by each generation of the host *de-novo* from the environment. In contrast, **heritable** bacterial symbionts are transmitted vertically from the host parent to the next generation of the fungal-bacterial metaorganism. Vertical transmission can be either strict/exclusive, or mixed, *i.e.* punctuated by instances of horizontal transmission in which bacteria spread between host individuals of the

77 same generation. Bacterial symbionts can be free-living. They can also be confined to their
78 eukaryotic host's intracellular environment and have no extracellular state (**obligate**
79 **endobacteria**), or capable of living both in fungal cells and in extracellular environments
80 (**facultative endobacteria**). Finally, mutualistic symbionts can be divided based on their effects
81 on host survival into **essential** and **nonessential**.
82 Because of varying levels of integration and complexity, understanding of fungal-
83 bacterial metaorganisms is at present in its infancy. We believe that many facets of this
84 biological complexity can be studied and framed conceptually using the existing ecology and
85 evolution tools and theory. For example, some spontaneously formed fungal-bacterial
86 associations can be explained by **ecological fitting**, in which organisms establish novel relations
87 with other species thanks to the traits that they already possess when they encounter their new
88 partners (Janzen 1985). Such relationships often develop in man-made or disturbed
89 environments. Other interactions are expected to be products of prolonged reciprocal selection
90 that tie individual partner taxa or guilds of interacting partners into ecologically and
91 evolutionarily stable alliances. One of the approaches for organizing the knowledge on how
92 these entities are structured internally and coexist in ecosystems involves reconstruction of
93 **symbiotic networks** to inventory and display interactions among taxa within and across different
94 metaorganisms. In addition to being an inventory of taxa and their interactions, the networks are
95 expected to offer insights into the coevolutionary processes that shape the diversity of both
96 metaorganism constituents and metaorganisms themselves (Bascompte and Jordano 2013). In
97 particular, they represent patterns of selection operating among genetically variable multi-species
98 groups in which the species convergently adapt and specialize on a suite of symbiotic traits
99 rather than directly on other species (Thompson 2005). While, historically, symbiotic networks

100 have been used to represent interactions in mutualisms (Bascompte and Jordano 2013), they can
101 also accommodate interactions with negative fitness outcomes. Another framework that can help
102 explore and conceptualize fungal-bacterial interactions is the **geographic mosaic of coevolution,**
103 **GMC,** model (Thompson 2005). According to this model, partners interact across their
104 geographic ranges. In some locations, known as **coevolutionary hot spots**, they are subjected to
105 reciprocal selection. In others, known as **coevolutionary cold spots**, local selection is not
106 reciprocal. Several factors, including gene flow, genetic drift, mutations, migration, and local
107 extinctions, contribute to variation in the patterns of natural selection between the habitats.
108 These predictions can be readily translated into set of questions to guide investigations of fungal-
109 bacterial interactions (Gomulkiewicz et al. 2007).
110 While many fungal-bacterial interactions remain ambiguous in terms of fitness outcomes,
111 the vast majority of them are either undisputed antagonisms or mutualisms. The astounding
112 ubiquity and prevalence of antagonistic interactions present in all ecosystems is related to the
113 fact that living organisms represent excellent sources of energy and nutrients, which otherwise
114 are available in limiting quantities (Thompson 2014). In fact, even mutualisms are viewed as
115 reciprocal exploitations that nonetheless provide net benefits to each partner (Herre et al. 1999).
116 Moreover, despite their fundamental significance to the evolution and functioning of the
117 biosphere, the mechanisms that promote the initial establishment and evolutionary stability of
118 mutualisms are not fully explored. Like antagonisms, mutualisms can form instantaneously as a
119 consequence of ecological fitting (Janzen 1985; Hom and Murray 2014). They can be also
120 products of extensive reciprocal selection between the partners that initially interacted as either
121 antagonists or commensals (Aanen and Bisseling 2014). Conflicting interests of the interacting
122 partners, manifested by accepting benefits without reciprocating, make mutualisms vulnerable to

failures. Yet, their evolutionary persistence suggests that certain mechanisms could ensure mutualism stability (Trivers 1971). Several theoretical models have been proposed to explain evolutionary stability of mutualisms. They include: (1) **byproduct cooperation** (Connor 1986; Sachs et al. 2004), (2) the **iterated prisoner's dilemma**, **IPD**, model with the **"tit-for-tat"** strategy (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs et al. 2004), (3) **partner-fidelity feedback**, **PFF** (Bull and Rice 1991; Sachs et al. 2004; Weyl et al. 2010), (4) **partner choice** (Bull and Rice 1991; Noë and Hammerstein 1994; Sachs et al. 2004), and (5) **compensatory evolution/addiction** (Aanen and Hoekstra 2007). (1) Byproduct cooperation involves interactions in which a focal partner receives a byproduct benefit from a donor and natural selection shapes the focal partner to maximize these benefits by being cooperative toward the donor (Connor 1986; Sachs et al. 2004). (2) The IPD model with the "tit-for-tat" strategy applies to systems in which two partners, who engage in a series of interactions, are able to vary their behavior in each interaction according to a partner's previous action (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs et al. 2004; Weyl et al. 2010). Cooperation is maintained only when partners reciprocate in kind. Non-cooperative individuals are sanctioned by their partners through termination of cooperation. (3) Like IPD, the PFF model applies to systems in which two partners interact repeatedly. However, in PFF, fitness gains derived from cooperation by one partner feed back to the other partner, thus the partner who fails to cooperate harms its own fitness (Bull and Rice 1991; Sachs et al. 2004; Weyl et al. 2010). (4) Unlike IPD and PFF, the partner choice model involves interactions of a focal individual with multiple trading partners who are reciprocated based on the quality of goods and services offered, with the most cooperative partner receiving the highest compensation (Sachs et al. 2004; Kiers et al. 2011). (5) The mechanism of compensatory evolution/addiction is expected to operate in

mutualisms that evolved from antagonistic interactions, in host populations exposed initially to a parasitic symbiont (Aanen and Hoekstra 2007). Under parasite pressure, host mutants are favored that compensate for harmful effects of the parasite and thus suffer less damage. Once such compensatory mutations are fixed, they may become deleterious to the host in the absence of the parasite. As a consequence, a host population with such compensatory mutations will become dependent on the presence of the parasite, leading ultimately to a conversion of an antagonistic interaction into a stable mutualism.

For the sake of clarity, we divided our discussion of fungal-bacterial symbioses into sections devoted to systems in which partners are assembled *de novo* in each generation versus associations in which partners are transmitted together from generation to generation and interactions are heritable. We also discussed the role of vertical transmission in evolution of mutualisms from antagonisms. Finally, we suggested tools and future directions for studying fungal-bacterial symbioses.

III. Non-Heritable Symbiotic Interactions

A. Introduction

All basic types of relationships, *i.e.* mutualisms, commensalisms, and antagonisms, can be found among non-heritable fungal-bacterial symbioses. For some of them detailed knowledge is available, others will be mentioned only briefly. Some bacteria associate directly with fungal hyphae (Baschien et al. 2009; Cuong et al. 2011) and form biofilms on their surfaces (Simon et al. 2015; Pion et al. 2013; Scheublin et al. 2010). These epibionts live in the hyphosphere, the volume around hypha influenced by the hyphal presence (**Errore. Riferimento a collegamento ipertestuale non valido.**). The bacterial symbionts can be antagonistic, as is

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typical for bacteria used for biocontrol of fungal pathogens (Mela et al. 2011; Cuong et al. 2011; Jochum, Osborne, and Yuen 2006; Mathioni et al. 2013). They can also act as mutualists (Nazir, Tazetdinova, and van Elsas 2014). However, it seems that there is a limited number of fungiphilic bacterial taxa, *i.e.* taxa adapted to the mycosphere, that are involved in fungal-bacterial symbioses (Lyons et al. 2005; Warmink, Nazir, and van Elsas 2009; Simon et al. 2015; Baschien et al. 2009; Scheublin et al. 2010). Finally, some non-heritable interactions are quite unexpected and thought provoking, like those formed by bacteria-farming fungi (Pion et al. 2013), or bacterivorous nematodes and nematophagous fungi (Wang et al. 2014; Hsueh et al. 2013).

B. *Candida albicans-Pseudomonas aeruginosa* antagonism

Because of their significance to human health, interactions between *Candida albicans* and *Pseudomonas aeruginosa* attracted a lot of attention, which, in turn, yielded important insights into the molecular mechanisms that underlie the coexistence of these two organisms in the context of human disease (Peleg, Hogan, and Mylonakis 2010). *C. albicans* is a commensal yeast found in the normal microbial flora of human oral, digestive, or vaginal mucosa (McManus and Coleman 2014). It is acquired at birth or during physical contact. Factors affecting the mucosal microbiome, such as the use of antibiotics, hormonal imbalance, or diet, can induce non-life threatening *C. albicans* infections of mucosal surfaces, candidiasis (Scully, el-Kabir, and Samaranayake 1994). In severely ill and immunocompromised individuals, *C. albicans* can spread into the blood stream causing invasive and often fatal candidaemia (Eggimann, Garbino, and Pittet 2003). *C. albicans* invasions of host tissues are associated with a morphogenic switch from yeast-like to filamentous growth, which can be induced by changes in environmental

192 conditions, such as shifts in temperature and pH (Berman and Sudbery 2002).

193 *C. albicans* history is intimately linked with the history of humans. Phylogenetic data
194 suggest that its diversification occurred ~3 to 16 MYA and coincided with the evolution of early
195 hominids (Lott et al. 2005). Moreover, it is believed that humans are the main environmental
196 reservoir of *C. albicans* (Angebault et al. 2013). In contrast to *C. albicans*, *P. aeruginosa* is a
197 ubiquitous microbe that can be isolated from diverse environments, including humans (Lister,
198 Wolter, and Hanson 2009). However, unlike *C. albicans*, it is rarely a member of the normal
199 microbial flora in humans. Instead, it is a causal agent of community-acquired and, more often,
200 nosocomial infections in individuals who are immunocompromised or suffered a breach in
201 cutaneous or mucosal barriers. The recently observed rise in opportunistic *P. aeruginosa*
202 infections appears to be related to the ability of this microbe to rapidly develop multidrug-
203 resistant phenotypes.

204 Mixed infections in which *P. aeruginosa* coexists with *C. albicans* often occur in patients
205 with burn wounds (Gupta et al. 2005) and chronic lung diseases (Hughes and Kim 1973). In
206 such infections the two organisms display an array of antagonistic interactions centered on
207 competition for the host resources and mediated by several mechanisms. For example, *C.*
208 *albicans* responds to the *P. aeruginosa* quorum-sensing signal 3-oxo-C12 homoserine lactone
209 (3OC12HSL) as well as its 12 carbon chain analogs C12HSL and dodecanol with the inhibition
210 of yeast cell filamentation and conversion of previously formed filaments to yeast cells (Hogan,
211 Vik, and Kolter 2004). These are likely defensive responses, as *P. aeruginosa* can attach to the
212 surface of *C. albicans* hyphae and kill them through the action of phospholipase C and
213 phenazines; yeast cells are not susceptible to *P. aeruginosa* attachment (Hogan and Kolter 2002;
214 Gibson, Sood, and Hogan 2009).

Initially, the morphogenic effects of *P. aeruginosa*-derived C12 compunds on *C. albicans* were considered to be purely coincidental as these molecules share structural similarity with farnesol. Farnesol is the C12 autoregulatory molecule that controls yeast-to-hypha transition in *C. albicans* (Hogan, Vik, and Kolter 2004) by modulating cyclic AMP signaling through direct inhibition of the adenylate cyclase activity (Davis-Hanna et al. 2008; Lindsay et al. 2012; Hall et al. 2011) and suppressing filamentation of yeast cells (Hornby et al. 2001). Recent studies revealed that despite structural similarities among the C12 HSLs and their analogs, only 3OC12HSL mimics farnesol's activity by interacting with the adenylate cyclase. Another C12 compound, dodecanol prevents yeast-to-hypha transition through a different mechanism involving the transcriptional hyphal suppressor Sfl1p (Hall et al. 2011). Interestingly, dodecanol shares structural similarity with a diffusible signal factor of *Burkholderia cenocepacia* (Hall et al. 2011), which also interferes with *C. albicans* filamentation (Boon et al. 2008). Like *P. aeruginosa*, representatives of the *B. cepacia* complex frequently coexist and interact antagonistically with *C. albicans* in mixed infections of patients who are immunocompromised and suffer chronic lung disease (Kerr 1994). Notably, however, *C. albicans* does not seem to respond to C8 HSL, the major quorum-sensing signal produced by *B. cepacia* (Hogan, Vik, and Kolter 2004; Boon et al. 2008).

In addition to autoregulation of fungal morphogenesis, farnesol plays a role in interactions with bacterial antagonists by inhibiting biosynthesis of the *P. aerugionosa* quinolone signal (PQS) and the PQS-controlled biosynthesis of the pyocyanin siderophore virulence factor (Cugini et al. 2007). Moreover, *C. albicans* interferes with *P. aeruginosa* signaling and metabolite production. It can also inhibit virulence of *P. aeruginosa* in mice by inhibition of bacterial pyochelin and pyoverdine siderophore biosynthesis (Lopez-Medina et al. 2015).

While the structural similarity of compounds that suppress yeast-to-hypha transition in *C. albicans* may suggest ecological fitting, the diversity of the morphogenic mechanisms utilized by *C. albicans* to respond to these bacterial C12 signal molecules as well as the complex interplay of inhibitory interactions between *C. albicans* and its bacterial antagonists suggest that these organisms may have been undergoing reciprocal selection within the context of human disease. This process is expected to intensify with the continued increase in the number of patients who require immune system suppression.

C. Mycophagy and biological control of fungi by bacteria

Fungal hyphae are a potential nutrient and energy source for bacteria. Some bacteria seem to be specialized in feeding on fungi and have been considered mycophagous (Leveau and Preston 2008). They have been studied mainly as potential biological control agents aimed toward plant pathogenic fungi (Jochum, Osborne, and Yuen 2006; Yoshida et al. 2012; Selin et al. 2010). These antagonistic bacteria can kill the fungus using a combination of enzymes and antifungal compounds. A well-studied and interesting antifungal compound produced by *Lysobacter* is HSAF (heat-stabile antifungal factor), a hybrid PKS-NRPS inhibiting the fungal acyl-CoA-dependent ceramide synthase, an enzyme unique to filamentous fungi (Li et al. 2008; Yu et al. 2007). This inhibition affects the formation of lipid rafts that are important for proper fungal exocytosis and endocytosis (Li et al. 2006; Alvarez, Douglas, and Konopka 2007).

Importantly, most potential biological control organisms have been selected for their ability to produce antifungal compounds on agar plates but it is unclear if they also use the fungus as an energy or carbon source or, indeed, if the same inhibiting compounds are active as biocontrol agents in the natural environments (Thrane et al. 2000). Moreover, it is not necessary

for mycophagous bacteria to lyse the fungal hyphae in order to parasitize the fungus, proliferate, and inhibit the fungus efficiently. Some bacteria kill the fungus and multiply without penetrating its cell walls, while others proliferate without any negative effects to the fungus (Cuong et al. 2011).

With the advent of transcriptomics and proteomics, new insights have been gained into these antagonistic of interactions. For example, dual transcriptomic studies of both the fungus and the bacterium challenging each other on agar plates focused on interactions between *Aspergillus niger* and *Collimonas fungivorans* (Mela et al. 2011) as well as *Rhizoctonia solani* and *Serratia plymuthica* (Gkarmiri et al. 2015; Neupane et al. 2015). In these studies, the partners were not allowed to come into physical contact but could exchange metabolites, and in both cases the portion of the fungal colony that was transcriptionally profiled was the one adjacent to the inhibition zone. Both studies found that the fungi reacted by upregulating defense responses (detoxification, efflux pumps), changes to membrane permeability, and increased oxalate production. In contrast, the only response common in bacteria was the upregulation of genes involved in production of secondary metabolites (Mela et al. 2011; Gkarmiri et al. 2015). The two interactions were in many other ways quite different. The *Aspergillus*-*Collimonas* interaction was mainly characterized by a competition for nitrogen (Mela et al. 2011), while the *Rhizoctonia*-*Serratia* interaction involved a mutual chemical warfare, as both the fungus and the bacteria upregulated transcription of genes responsible for secondary metabolites/toxins and defenses (Gkarmiri et al. 2015; Neupane et al. 2015).

Another example of fungal-bacterial antagonistic interactions comes from *Magnaporthe oryzae* transcriptional responses after direct contact with *Lysobacter enzymogenes*, both a wild type (WT) strain and a mutant strain deficient in virulence (Mathioni et al. 2013). Four

Magnaporthe genes were induced at 3 hours by both WT and mutant bacteria, and two of these were known stress response genes (a laccase and a beta-lactamase). The hypothesis that WT *L. enzymogenes* is capable of turning off fungal defenses while the mutant could not was used to interpret the data. A total of 463 *Magnaporthe* genes were down-regulated by WT *L. enzymogenes*. Of these genes, 100 were up-regulated in interaction with the non-virulent mutant and assumed to be genes involved in the fungal general response/defense against bacteria. These genes are predicted to have roles in carbohydrate metabolism, cellular transport and stress response (Mathioni et al. 2013).

The examples discussed above offer glimpses into the vast and complex world of metabolic activities involved in trophic interactions between bacteria and fungi, as we are only starting to uncover and understand these food webs. Clearly, further sustained efforts are needed to identify the players and understand the flows of energy and nutrients that support the communities of fungi and bacteria forming such trophic networks.

D. Fungal predation and dependence on bacteria

Fungi can attack, degrade, and use bacteria as nutrient sources (Barron 1988; Barron 2003). These capabilities have mainly been noted in basidiomycete wood decomposers, with nitrogen limitation being the main trigger of fungal predation on bacteria (Barron 2003). Wood decomposing fungi have profound effects on bacterial composition of the substrate they colonize and the bacterial composition becomes characteristic for the fungal species colonizing the substrate (Tornberg, Bååth, and Olsson 2003; de Boer et al. 2005). Along similar lines, nitrogen fixation by bacteria seems to be important in wood decay and it has been suggested that nitrogen-fixing bacteria grow on the low molecular carbon released by the wood decaying fungi and that

307 the fungus then selectively harvests and degrades some of the bacteria as a source of nitrogen (de
308 Boer and van der Wal 2008). This idea has found support in a study of the *nifH* dinitrogenase
309 reductase diversity in dead wood, where a non-random co-occurrence pattern between nitrogen-
310 fixing bacteria and fungal species was detected, indicating specific interactions between fungi
311 and bacteria (Hoppe et al. 2014). Similarly, *Rhizobium*-type nitrogen-fixing bacteria can form
312 biofilms on fungi and this seems to affect the activity and survival of both organisms
313 (Seneviratne and Jayasinghearachchi 2003; Seneviratne et al. 2008).

314 Of relevance to the observations on the trophic interactions between fungi and bacteria is
315 the concept of bacteria farming by fungi, which was recently introduced to describe the
316 relationship between the fungus *Morchella crassipes* and *Pseudomonas putida* (Pion et al. 2013).
317 *M. crassipes* disperses bacteria, rears them on fungal exudates as well as harvests and
318 translocates bacterial carbon (Pion et al. 2013). It is possible that a similar mechanism of
319 bacteria farming by fungi can be behind the observed interactions between nitrogen-fixing
320 bacteria and fungi and could account for the apparent stability of the interactions.

321 Finally, not all trophic interactions involving fungi and bacteria are antagonistic. An
322 example of a more complex interaction comes from the cow dung-inhabiting bacterium
323 *Stenotrophomonas maltophila.* These bacteria are consumed by the bacterivorous nematode
324 *Caenorhabditis elegans.* As a defense mechanism, the bacteria secrete urea that mobilizes the
325 nematophagous fungus *Arthrobotrys oligospora* to respond to the nematode presence and
326 eliminate them. Nematode elimination is accomplished by the increased production of sticky
327 hyphal nets that trap and kill nematodes, which are then consumed by the fungus (Wang et al.
328 2014; Hsueh et al. 2013).

Like with trophic interactions in which bacteria feed on fungi, fungal predation and farming of bacteria are most likely widespread and underappreciated features of terrestrial ecosystems. While some of them can be readily reproduced under laboratory conditions, others need to be studied *in situ* in their natural environments to understand how they connect to more conventional food webs.

E. Highways carrying hyphae-associated bacteria

Fungal hyphae expanding in and through unsaturated soil can spread in a soil volume easier than bacteria, as they can bridge over aerial pores and other hydrophobic regions (Kohlmeier et al. 2005). The surfaces of the fungus assimilatory hyphae are hydrophilic and thus the fungal hyphae form hydrophilic tracks through soil. These tracks are referred to as **fungal highways** that the bacteria can follow and are generally regarded as beneficial to both the host and the bacterial symbionts (Kohlmeier et al. 2005). The fungal highways have been studied in relation to dissemination of pollutant-degrading bacteria (Kohlmeier et al. 2005; Furuno et al. 2010). In particular, it has been shown that the fungal hyphae might not just help to spread the bacteria but could also function as conduits of pollutants to bacteria (Banitz et al. 2014; Furuno et al. 2010; Wick et al. 2007). In this respect, substrate is channeled from a source along the hyphae to bacteria that are associated with these hyphae. The fungal host seems to nourish the bacterial symbionts inhabiting and spreading on the highways (Bravo et al. 2013; Nazir et al. 2013). The number of bacterial taxa associating and travelling along the fungal highways is probably a combination of selection for the specific prevalent conditions, available substrates, and also by direct activities of the host, *e.g.* a consequence of mutualist recognition or absence of parasite recognition. Bacterial motility by flagella as well as other types of motility have been suggested

as a common characteristic of bacteria travelling on the fungal highways (Bravo et al. 2013). Among bacterial taxa especially common in the hyphosphere is the genus *Burkholderia* (Suárez-Moreno et al. 2012). Interestingly, the same genus is also prominent among fungal endosymbionts (see sections below). Fungus-derived oxalate and glycerol have been shown to feed both mutualistic and parasitic bacterial symbionts living and spreading on the fungal highways (Bravo et al. 2013; Nazir et al. 2013). It has also been shown that some bacteria that migrate as "hitchhikers" along fungal highways can only do this if other bacteria have paved the way for them (Warmink et al. 2011). Interestingly, such facilitation does not apply to all bacteria (Warmink et al. 2011). Thus there appear to be three categories of bacteria in relation to movement along fungal hyphae: (1) independent travelers that manage to set up the conditions with the fungal hosts necessary for travel, (2) hitchhikers dependent on the simultaneous presence of the independent travelers, and (3) non-travelers, either not having the properties, such as motility, to move along the fungal highway, or being inhibited by the fungal host and/or the first two types of travelers.

The potential importance of fungal highways to the soil bacteria suggests that these interactions may be a common and, until recently, overlooked feature of soil ecosystems. Consequently, the diversity of both fungi that serve as the thoroughfares and their bacterial travelers requires in-depth surveying. The approach of symbiotic network reconstruction appears to be a natural starting point for understanding the rules that govern highway usage. Importantly, while bacterial travelers clearly benefit from highway availability, as it improves their mobility in the soil and may offer a source of nourishment, it is unclear whether fungi receive any benefits from this interaction. Is it a mutualism or an interaction in which the fungal partner remains unaffected or perhaps even harmed?

F. Mycorrhiza helper bacteria

Mycorrhizal fungi form with the roots of terrestrial plants symbiotic associations of distinct morphologies and functions, collectively referred to as mycorrhizas (Smith and Read 2008). In the most common among them, ecto- and arbuscular mycorrhizas, fungi facilitate plant mineral nutrient uptake from the soil in return for photosynthetic carbon. As a consequence, these symbioses are of great significance in both natural and managed ecosystems, with a particular impact on agriculture and forestry. Current observations indicate that mycorrhizas are, in fact, complex multipartner interactions (Bonfante and Anca 2009), due to the presence of bacteria that can be either loosely or tightly associated with mycorrhizal fungi (Jansa, Bukovská, and Gryndler 2013; Bianciotto et al. 2001; Perotto and Bonfante 1997). Garbaye (1994) pioneered the work on these associations with the now widely accepted term **mycorrhiza helper bacteria, MHB**, which defines bacteria that help mycorrhizal establishment. Since the time of MHB discovery and thanks to the advent of the omics era, new knowledge and insights have accumulated, with a particular focus on the microbiota present in the rhizosphere and endosphere of poplar (*Populus*).

As a host for both ecto- and arbscular mycorrhizal fungi (AMF), poplar is an excellent model for understanding interactions that govern establishment and functioning of mycorrhizal symbioses, including the role of MHB. For example, the genomes of 21 strains of *Pseudomonas* isolated from the *Populus deltoides* rhizosphere and endosphere have been sequenced (Brown et al. 2012), giving rise to extensive genetic and bioinformatic resources. As a further step, these bacterial isolates were screened for MHB effectiveness expressed as the effects on the *Laccaria bicolor* S238N growth rate, mycelial architecture, transcriptional changes and symbiosis with

three *Populus* lines, *P. tremula* × *alba*, *P. trichocarpa*, and *P. deltoides*. Nineteen of the studied isolates had positive impact on *L. bicolor* growth (Labbé et al. 2014). Interestingly, one strain promoted high root colonization also in *P. deltoides,* which is otherwise poorly colonized by *L. bicolor*. In this context, the genome of a MHB isolate of *Pseudomonas fluorescens* BBc6R8 will be of great advantage in identifying the helper traits (Deveau et al. 2014).

Prokaryotes are associated not only with the extraradical hyphae of mycorrhizal fungi, but also with ectomycorrhizal roots and sporocarps, *i.e.*, the fruiting bodies formed by ectomycorrhizal ascomycetes and basidiomycetes, suggesting that they may accompany mycorrhizal fungi during the various steps of their life cycle. Because of their economic significance, *Tuber* sporocarps have become a model to understand a role that truffle-associated bacteria play in several still poorly understood aspects of truffle development, from fruiting body formation to aroma production. Similarly, the appearance of the "brûlé", an area devoid of vegetation around the *Tuber* host plants and where the fruiting bodies of *T. melanosporum* are usually collected, is a feature with a clear ecological impact but largely unknown causes. For example, the examination of direct fungal-fungal interactions (Napoli et al. 2010), together with DGGE and DNA microarray analyses of 16S rRNA gene fragments (Mello et al. 2013), revealed that the bacteria and archaeal communities strongly differ between the inside versus outside of the brûlé area. The groups that were most severely affected by the black truffle included Firmicutes, several genera of Actinobacteria, and a few Cyanobacteria. One of the mechanisms responsible for this pattern could be the capacity of truffles to release volatile organic compounds (Splivallo et al. 2011). Intriguingly, Splivallo et al. (2015) found that sulphur-containing volatiles, such as thiophene derivatives characteristic of *T. borchii* fruiting bodies, are products of the bacteria-mediated biotransformation of non-volatile precursor(s) into volatile

compounds. Moreover, the α- and β-proteobacteria-dominated community of *T. borchii* was able to produce thiophene volatiles from *T. borchii* fruiting body extract, irrespective of their isolation source (truffle or other sources).

The complexity of interactions between fungi and both MHB and sporocarp-associated bacteria makes them uniquely difficult to study. However, the tools of symbiotic network construction and testing the applicability of the GMC model to these systems may provide structured approaches to make rapid progress in understanding of these systems.

G. Recognition and assembly of the non-heritable symbionts to form the fungal-bacterial metaorganism

Both plant and animal epithelial surfaces coming in contact with bacteria share a similar problem in that they should actively select for beneficial/commensal bacteria and discourage the colonization by antagonists (McFrederick et al. 2012; Artis 2008; Ausubel 2005; Zamioudis and Pieterse 2012). Innate immunity recognition of bacterial cues as MAMPs (microbial associated molecular patterns) plays a key role in this selection in both plants and animals (Artis 2008; Nürnberger et al. 2004). However, the immune reaction is balanced so as not to kill eventual beneficial bacteria, as is done in tissues not normally colonized by bacteria (Artis 2008; Zamioudis and Pieterse 2012). Fungal hyphae growing in most natural environments face a similar need to promote the beneficial and inhibit the antagonistic microbes. Fungal reactions to a bacterial MAMP have been demonstrated (Xu et al. 2008), the existence of innate immunity type recognition has been suggested (Paoletti and Saupe 2009; Paoletti, Saupe, and Clavé 2007), and recently transcriptomic innate immunity type responses have been found in fungi (Ipcho et al. 2016). Fungal innate immunity is thus most likely involved in the active selection for

444 beneficial bacteria as it is in other eukaryotic hosts. The main mechanisms of such selection
445 involve production of antibiotics/secondary metabolites, selective provisioning of nutrients to the
446 beneficial bacteria (Huang et al. 2014; Hartmann et al. 2009; Ramírez-Puebla et al. 2013; Oozeer
447 et al. 2013; Scholtens et al. 2012), and creating conditions unfavorable for pathogens (Kai-
448 Larsen, Gudmundsson, and Agerberth 2014; Markel et al. 2007; Ramírez-Puebla et al. 2013).
449 The selective recruitment of beneficial bacteria is further helped by their either passive or active
450 transfer between host generations (Oozeer et al. 2013; Scholtens et al. 2012; Ramírez-Puebla et
451 al. 2013), thus resembling heritable transmission.

452 Interestingly, several mechanisms appear to be shared by diverse host symbiont-systems
453 (Table 1). For example, the gut epithelium, the rhizoplane, and the hyphosphere are typically
454 low-pH environments and this pH decrease is stimulated further by bacterial presence (Ramírez-
455 Puebla et al. 2013), a condition also shared by animal tissue inflammation (Rajamäki et al.
456 2013). Another key reaction in innate immunity is active sequestration of iron by the plant and
457 animal hosts (Markel et al. 2007; Ganz 2009; Ong et al. 2006; Lemanceau et al. 2009). As a
458 consequence, iron levels are much depleted both in the rhizosphere (Lemanceau et al. 2009) and
459 in the gut (Ganz 2009; Markel et al. 2007; Ong et al. 2006). Beneficial bacteria appear to be
460 adapted to such low-iron conditions and display either very low demand for iron, as the probiotic
461 *Lactobacillus plantarum* (Archibald 1983), or have very efficient siderophores, like many plant
462 growth-promoting rhizobacteria (Beneduzi, Ambrosini, and Passaglia 2012). Interestingly, most
463 genes involved in iron acquisition are also rapidly upregulated in *Fusarium graminearum* in
464 response to bacterial MAMPs (Ipcho et al. 2016). Finally, beneficial bacteria in the rhizosphere
465 are stimulated by plant rhizosphere-specific sugars, like raffinose and sucrose (Huang et al.
466 2014), which are generally not present in the soil, while beneficial gut bacteria in mammals are

467 stimulated by fructans (Oozeer et al. 2013; Scholtens et al. 2012). Fungi interacting with
468 bacteria have been also shown to secrete carbon sources, such as oxalate (Scheublin et al. 2010),
469 glycerol (Nazir et al. 2013) and trehalose (Deveau et al. 2010), which could possibly serve
470 similar selective functions for beneficial bacteria.
471 The mechanisms responsible for the assembly of fungal-bacterial metaorganisms thus
472 appear to have parallels with other eukaryotic-bacterial metaorganisms and much can be learnt
473 from these other systems. Because fungi are relatively easy to study and manipulate genetically,
474 there is a great potential for rapid progress in understanding the fungal-bacterial interactions.
475 Importantly, we expect that all horizontally transmitted bacterial symbionts as well as the
476 bacteria engaged in heritable facultative mutualisms with fungi need to employ these
477 mechanisms when initiating the interaction with their hosts.
478
479 **IV. Vertical Transmission and the Evolution of Mutualisms**
480 Because of its role in coupling of partner reproductive interests, vertical transmission is widely
481 recognized as a mechanism that stabilizes mutualisms under several evolutionary models,
482 including byproduct cooperation (Connor 1986; Sachs et al. 2004), IPD with the "tit-for-tat"
483 strategy (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs et al. 2004), PFF (Bull
484 and Rice 1991; Sachs et al. 2004; Weyl et al. 2010), and compensatory evolution/addiction
485 (Aanen and Hoekstra 2007). In addition, vertical transmission is expected to play an important
486 part in evolution of antagonistic interspecific interactions into mutualisms (Yamamura 1993).
487 Evolutionary theory predicts that a symbiotic system will transition from antagonism to
488 mutualism once a parasite is able to dominate the co-evolutionary race with the host and achieve
489 a rate of vertical transmission that enables efficient reciprocal selection between the partners

(Yamamura 1993) (Fig. 2). If the increase in the rate of symbiont vertical transmission is accompanied by the development of host abilities to complement its metabolism using symbiont metabolites, a byproduct mutualism is expected to evolve (Yamamura 1993).

While the model that explains the evolution of mutualisms from antagonisms through changes in the rates of symbiont transmission is rather straightforward (Yamamura 1993), the actual mechanisms that permit symbiont vertical transmission remain elusive as nearly all known heritable endosymbionts are uncultivable (Moran, McCutcheon, and Nakabachi 2008) and many hosts are unable to survive without their endobacteria. In this context, the rice seedling blight fungus *Rhizopus microsporus* and its endosymbiont *Burkholderia rhizoxinica* offer an unprecedented opportunity to understand the evolution of mutualisms from antagonisms (Partida-Martinez and Hertweck 2005; Lackner and Hertweck 2011). In this system, the endobacteria reside directly within the fungal cytoplasm (Partida-Martinez et al. 2007). Their elimination with antibiotics abolishes fungal ability to form asexual sporangia and sporangiospores (Partida-Martinez et al. 2007), suggesting that endobacteria not only gained control of their own transmission rate but also of the reproductive success of the fungus, a pattern consistent with the compensatory evolution/addiction model of mutualism evolution (Aanen and Hoekstra 2007). In addition to controlling the rate of own vertical transmission by rendering fungal reproduction dependent on their presence, the endobacteria produce a macrolide metabolite that is processed by the host to form a highly potent antimitotic toxin called rhizoxin (Scherlach et al. 2012). The toxin is active in rice seedlings, where it causes the blight disease (Lackner, Partida-Martinez, and Hertweck 2009). In addition, rhizoxin is believed to facilitate competitive interactions of the *Rhizopus* host with fungi that are sensitive to it. Such positive effects of the symbiont-derived metabolite on host fitness suggest that the *Rhizopus*-

513 *Burkholderia* symbiosis can be viewed as a byproduct mutualism, in addition to being an
514 example of the addiction model. The *Rhizopus* host, like other Mucorales, is protected from
515 harmful effects of the toxin by a specific mutation in its β-tubulin gene (Schmitt et al. 2008).
516 The presence of this protective mutation across other Mucorales suggests that it was a
517 preadaptation that allowed *Rhizopus* for entering a byproduct mutualism with the *Burkholderia*
518 endobacteria.

519 The 3.75 Mb genome of *B. rhizoxinica* appears to be moderately sized compared to free-
520 living *Burkholderia* with genomes of 8 – 9 Mb (Winsor et al. 2008), but is considerably larger
521 than the genomes of closely related endosymbiotic β-proteobacteria, including *Candidatus*
522 Glomeribacter gigasporarum (1.72 Mb) (Ghignone et al. 2012), the unnamed endosymbiont of
523 *Mortierella elongata* (2.65 Mb) (Fujimura et al. 2014), and *Ca.* Tremblaya princeps (0.14 Mb)
524 (McCutcheon and von Dohlen 2011). Such reductions in the endosymbiont genome size are
525 associated with the process of adaptation to the host cellular environment (McCutcheon and
526 Moran 2012). Nevertheless, the *Burkholderia* endobacteria of *Rhizopus* remain not only
527 metabolically independent of the host and but also capable of invading compatible hosts *de novo*
528 (Moebius et al. 2014). In particular, the release of bacterial chitinolytic enzymes and chitin-
529 binding proteins enables breaching of fungal cell walls and the initiation of the invasion process
530 (Moebius et al. 2014). In turn, the survival and proliferation of *B. rhizoxinica* inside fungal cells
531 appears to depend on the activity of the type III secretion system (Lackner, Moebius, and
532 Hertweck 2011), and the presence of a specific O-antigen in the lipopolysaccharides, LPS, that
533 make up the outer membrane of these Gram-negative bacteria (Leone et al. 2010). It is not
534 affected, however, by the structural changes in the exopolysaccharide, EPS, secreted matrix
535 (Uzum et al. 2015).

Even though some of the features displayed by the *Rhizopus-Burkholderia* symbiosis are typical for a mutualism, the *Burkholderia* endobacteria appear to be facultative endosymbionts, capable of living both inside and outside eukaryotic cells, a lifestyle similar to that of pathogenic *Legionella*, *Salmonella*, or *Bartonella*. This duality, combined with the ease of experimental manipulation, propelled the *Rhizopus-Burkholderia* symbiosis to become a model for studying the evolution of heritable symbioses. In particular, addressing questions concerning its evolutionary origins, whether it started with the partners interacting as antagonists (Fig. 2), and whether it has already achieved evolutionary stability (Fig. 3) will be a source of rich insights not only into the genetic mechanisms of symbiont vertical transmission but also into other facets of partner coevolution.

V. Heritable Symbiotic Interactions

A. Introduction

As discussed in the preceding sections, symbiont vertical transmission is a principal factor contributing to both the establishment and stability of mutualisms. Importantly, vertical transmission is not exclusive to mutualisms; it can also occur in antagonistic interactions. Vertical transmission can be strict or mixed. In strict vertical transmission symbionts are transferred from a parent exclusively to offspring. In mixed transmission, in addition to being passaged between generations, symbionts move horizontally between members of the same generation. Symbioses with strict vertical transmission are characterized by congruity of partner phylogenetic histories, consistent with partner codiversification (Page 2003). In symbioses with mixed transmission, the extent of horizontal transmission determines the degree of incongruity between partner phylogenies. Interestingly, strict vertical transmission of symbionts tends to be

559 associated with reciprocally obligate partner dependence, whereas mixed transmission is found
560 in associations in which either one or both partners are facultatively dependent on the symbiosis
561 (Fig. 3).

562 Importantly, while in fungi all known heritable associations involve endobacteria that
563 reside inside fungal cells, not all associations formed by fungi with endobacteria are known to be
564 heritable. In heritable symbioses, bacteria are either facultatively or obligately dependent on the
565 fungus. The *Burkholderia* symbionts of *Rhizopus,* discussed in the previous section, as well as
566 *Rhizobium radiobacter* in the root-colonizing *Piriformospora indica* (Sharma et al. 2008)
567 represent facultative heritable endobacteria. In contrast, obligate heritable endosymbionts
568 include two groups of bacteria associated with AMF, *Ca.* Glomeribacter gigasporarum
569 (Bianciotto et al. 2003) and the mycoplasma-related endobacteria, MRE (Naumann, Schüßler,
570 and Bonfante 2010). It is unclear whether the unnamed heritable endosymbiont of *Mortierella*
571 *elongata* (Sato et al. 2010) is a facultative or obligate endobacterium. Remarkably, we are not
572 aware of heritable fungal-bacterial symbioses in which the interacting partners are obligately
573 dependent on each other. Such associations are common in insects, which depend on
574 endobacteria for provision of essential nutrients (McCutcheon and Moran 2012). It remains to
575 be investigated whether this knowledge gap represents a true dearth of reciprocally obligate
576 fungal-bacterial interactions or a detection bias. Recent accumulation of newly discovered
577 associations that involve non-heritable endobacteria suggests that the latter might be the case.
578 Such non-heritable associations include, among others, *Helicobacter pylori* in *Candida albicans*
579 (Siavoshi and Saniee 2014), *Nostoc punctiforme* in *Geosiphon pyriforme* (Schüßler et al. 1994),
580 *Bacillus* spp. in *Ustilago maydis* (Ruiz-Herrera et al. 2015), α-proteobacteria in the
581 ectomycorrhizal fungus *Laccaria bicolor* (Bertaux et al. 2005; Bertaux et al. 2003), and diverse

582 bacteria that inhabit hyphae of phylogenetically diverse fungal endophytes of plants (Hoffman
583 and Arnold 2010). Due to the lack of sufficient data from other systems, our discussion in the
584 following two sections will focus on *Ca.* Glomeribacter gigasporarum and MRE associated with
585 AMF.
586
587 **B.** Heritable facultative mutualisms
588 *Ca.* Glomeribacter gigasporarum, referred hereafter as *Glomeribacter*, is a stable, and
589 structurally integrated endosymbiont found in many representatives of the AMF family
590 Gigasporaceae (Bianciotto, Bandi, et al. 1996; Bianciotto et al. 2003; Mondo et al. 2012). It
591 thrives inside the fungal cells along the different stages of the fungal life cycle, always located
592 inside a compartment structurally resembling a fungal vacuole (Bianciotto, Minerdi, et al. 1996).
593 On the fungal side, the Gigasporaceae, like other AMF, form symbiotic associations with roots
594 of many plants, and may proliferate also in the absence of the endobacteria (Lumini et al. 2007),
595 giving rise to an association that is obligate for the bacterial partner and facultative for the fungal
596 host. A similar disparity is true for all AMF, as they fully depend on their host plants for energy,
597 while plants may complete their life cycle in the absence of AMF.
598 While biodiversity studies have demonstrated that *Glomeribacter* is widespread, they
599 have not identified factors responsible for the evolutionary stability of the Gigasporaceae-
600 *Glomeribacter* symbiosis, which dates back to the early Devonian (Mondo et al. 2012). The
601 *Glomeribacter* genome sequencing revealed that this endobacterium has a reduced genome of
602 1.7 Mb (Ghignone et al. 2012), consistent with its uncultivable status (Jargeat et al. 2004). It
603 lacks metabolic pathways leading to important amino acids, but has many amino acid permeases
604 for uptake of nutrients from the fungus, as expected of an endobacterium that depends on its host

for nutrients and energy (Fig. 4). Interestingly, the whole operon for biosynthesis of vitamin B12 is present in the *Glomeribacter* genome, but it is not clear whether this might represent any benefit for the fungus. In contrast to animals, which use B12-dependent enzymes for methionine synthesis and methylmalonate metabolism, fungi and land plants rely on B12-independent enzymes for these pathways (Young, Comas, and de Carvalho 2015). Consistent with this expectation, the genome of a model AMF, *Rhizophagus irregularis,* encodes B12-independent enzymes (Tisserant et al. 2013).

While the significance of *Glomeribacter* to the AMF hosts could not be gleaned from its genomic sequence, the availability of a stable endosymbiont-free AMF *Gigaspora margarita* BEG34 line, designated as B(–), allowed for direct comparisons with the line containing the endobacterium, B(+). These comparisons revealed several differences, both phenotypic (Lumini et al. 2007) and transcriptional (Salvioli et al. 2016), that speak to the role of *Glomeribacter* in the AMF host. For example, the B(–) AMF line was able to colonize its plant host but was impaired in mycelial growth and spore production compared to the B(+) line (Lumini et al. 2007). Moreover, benefits of the endosymbiont presence appeared to extend to the plant host, as the phosphate measurements in *Lotus japonicus* plants revealed a statistically higher phosphate quantity in the symbiosis established by the B(+) versus the B(–) AMF line (Salvioli et al. 2016). In turn, the transcriptome analysis showed that the endobacterium had a stronger effect on the pre-symbiotic phase of the fungus, supporting earlier phenotypic observations that *Glomeribacter* promotes germ tube extension in the AMF host (Lumini et al. 2007; Salvioli et al. 2016). Coupling of transcriptomics with physiological and cell biology approaches demonstrated that the bacterium increases the AMF sporulation success, raises the AMF bioenergetic capacity, increasing ATP production, and elicits mechanisms to detoxify reactive

628 oxygen species (Salvioli et al. 2016). Moreover, application of the TAT (transactivator of
629 transcription) peptide to translocate the bioluminescent calcium reporter aequorin revealed that
630 the B(+) AMF line had a lower basal intracellular calcium concentration than the B(–) line,
631 indicating that the endobacterium affects a large number of fungal cell functions, including
632 calcium metabolism, consistent with a potential role as a storage compartment for intracellular
633 calcium. Finally, the fungal mitochondrion and its main metabolic pathways (ATP synthesis,
634 respiration) appear to be important targets of the bacterial presence. Interestingly, the AMF
635 mitochondria are also the first target of strigolactones, the plant hormones that play a key role in
636 plant-fungal signaling (Al-Babili and Bouwmeester 2015; Bonfante and Genre 2015). In the
637 experiments where the B(+) and B(–) AMF lines were treated with a synthetic strigolactone,
638 GR24, the bacteria seemed to react to strigolactones, in agreement with data demonstrating the
639 GR24 treatment induces bacterial cell division (Anca et al. 2009). All these experiments,
640 confirmed by an extensive proteomic analysis (Vannini et al. 2016), revealed that the bacterium,
641 directly or indirectly, affects the oxidative status of the fungus. Moreover, these benefits appear
642 to be transmitted to the host plants (Vannini et al. 2016).

643 Collectively, although *Glomeribacter* exacts a nutritional cost on the AMF, the symbiosis
644 appears to improve the fungal fitness by priming mitochondrial metabolic pathways and
645 provisioning AMF with the tools to face environmental stresses. These observations suggest that
646 evolutionary stability of the Gigasporaceae-*Glomeribacter* mutualism could be best explained by
647 the PFF model (Bull and Rice 1991; Sachs et al. 2004; Weyl et al. 2010), as, at present, there are
648 no indications that non-cooperative partners are sanctioned in this system, a pattern expected
649 under the IPD model (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs et al.
650 2004). Neither there is evidence for byproduct cooperation (Connor 1986; Sachs et al. 2004) or

651 compensatory evolution/addiction (Aanen and Hoekstra 2007).

652 Despite the remarkable progress made recently in understanding the Gigasporaceae-
653 *Glomeribacter* symbiosis, there are many outstanding questions. For example, it remains unclear
654 what factors keep this association from evolving towards reciprocally obligate partner
655 dependence predicted by evolutionary theory (Fig. 3). It could be speculated that the benefits to
656 the AMF host depend on the environmental context and the association may break up when the
657 cost of supporting the endosymbiont becomes prohibitive. This scenario would explain why the
658 endobacteria in the Gigasporaceae-*Glomeribacter* symbiosis appear to retain the potential to
659 transmit horizontally and exchange genes, attributes that may have contributed to their
660 evolutionary longevity (Mondo et al. 2012).

661

662 **C.** Heritable antagonisms

663 The symbiosis between AMF and MRE (mycoplasma related endobacteria) represents an
664 outstanding deviation from the molecular evolution patterns both expected by evolutionary
665 models and detected thus far in heritable endobacteria (McCutcheon and Moran 2012), including
666 *Glomeribacter* (Mondo et al. 2012). In particular, MRE display extraordinary intra-host
667 diversity of their 16S rRNA gene (Naumann, Schüßler, and Bonfante 2010; Desirò et al. 2014;
668 Desirò et al. 2015; Toomer et al. 2015) and genomic sequences (Naito, Morton, and Pawlowska
669 2015; Torres-Cortés et al. 2015). In part, this diversity could be attributed to a high mutation
670 rate, related to the loss of DNA repair machinery from the MRE genomes, combined with the
671 apparent activity of mechanisms contributing to genome plasticity, such as recombination
672 machinery and mobile genetic elements (Naito, Morton, and Pawlowska 2015; Naito and
673 Pawlowska 2016). While the mechanisms responsible for genome plasticity are not expected to

674 operate in heritable mutualists with strict vertical transmission, they have been detected in
675 mutualists with mixed transmission (McCutcheon and Moran 2012), including *Glomeribacter*
676 (Mondo et al. 2012). Notably, though, the extent of intra-host diversity displayed by MRE
677 exceeds vastly the diversity exhibited by mutualists with mixed transmission (Naito and
678 Pawlowska 2016). In fact, the co-ocurrence of MRE and *Glomeribacter* in several AMF allowed
679 for direct comparisons of their rRNA gene diversity revealing that, while MRE sequences
680 formed highly divergent sequence clusters, no diversity was apparent in *Glomeribacter* (Desirò
681 et al. 2014; Toomer et al. 2015). This disparity in molecular evolution patterns between MRE
682 and heritable mutualists with mixed transmission lead to the hypothesis that MRE may be
683 parasites of AMF (Toomer et al. 2015). This hypothesis is built on the predictions of
684 evolutionary models (Frank 1994, 1996, 1996) suggesting that hosts are expected to benefit from
685 reduced mixing of endosymbiont lineages because genetically uniform endosymbionts are less
686 likely to engage in competition that damages the host (Fig. 5). Bottlenecks imposed by vertical
687 transmission on symbiont populations reduce symbiont diversity inside host individuals, and
688 thus, vertical transmission is expected to limit destructive competition among symbionts for the
689 host resources. On the other hand, decline in symbiont relatedness within a host is predicted to
690 increase host exploitation and favor symbionts that are able to transmit horizontally to secure
691 new hosts.

692 While ascertaining whether MRE are antagonists or mutualists of AMF requires
693 empirical data, inferences about factors that contribute to evolutionary stability of the MRE
694 association with AMF can be made from the molecular evolution patterns evident in their
695 genomes. Given the high mutation rate apparent in MRE, it could be expected that they are
696 vulnerable to genomic degeneration and extinction (McCutcheon and Moran 2012). Yet, co-

divergence patterns between MRE and the two fungal lineages in which MRE occur, AMF and the *Endogone* lineage of Mucoromycotina, suggest that the AMF-MRE association may predate the divergence between these two lineages and thus be as old or older than the Gigasporaceae-*Glomeribacter* symbiosis (Desirò et al. 2015; Toomer et al. 2015). It has been postulated that the key factors that prevent MRE from extinction are the mechanisms responsible for genome plasticity in MRE, including the recombination machinery and mobile genetic elements, (Naito, Morton, and Pawlowska 2015; Naito and Pawlowska 2016). Despite these advances, MRE remain an elusive group of endobacteria. Not only their role in the AMF host biology but also the mechanisms of putative horizontal transmission require experimental evaluation.

VI. Future Developments

A. Introduction

The establishment and outcomes of the fungal bacterial interactions are most probably a result of chemical communication where a compound from one partner elicits a response with another compound from the other partner (Baruch et al. 2014; Piispanen and Hogan 2008; Xu et al. 2008; Badri et al. 2009; Nazir et al. 2010; Schroeckh et al. 2009; Sengupta, Chattopadhyay, and Grossart 2013). This is typical for "ping-pong" type communications, where a communication from one interacting partner draws a response from the other partner (Griffin 2012). The correct order of events in ping-pong communication, rather than unique metabolites, could be selective and instrumental in establishing the relationship (like a combinatorial lock). With the advent of modern omics, these ping-pong events could be studied using transcriptomics (Mela et al. 2011; Gkarmiri et al. 2015; Neupane et al. 2015; Mathioni et al. 2013), proteomics (Moretti et al. 2010), and aided with metabolomics, allowing for hourly resolution of events during the

720 establishment of the interaction. Although for multispecies bacterial communities colonizing
721 fungal hyphae this type of study is a major challenge, it would be possible to perform (Moretti et
722 al. 2012) and allow to test predictions of a theoretical model suggesting that complex microbial
723 communities could be stabilized by counteraction of antibiotic synthesis and degradation
724 conducted by different members of the community (Kelsic et al. 2015).
725
726 **B.** Novel tools to study fungal-bacterial metaorganisms
727 Recently developed technologies, like laser dissection and imaging mass spectrometry (IMS),
728 could be adapted to sample and analyze fungal-bacterial interaction at the microscopic level.
729 Laser dissection could be used to sample single bacterial cells or fungal nuclei from different
730 locations, and combined with single cell genomics/trancriptomics (Kang et al. 2015; Saliba et al.
731 2014; Teichert et al. 2012), reveal site-dependent activities of various bacteria. IMS (Watrous,
732 Alexandrov, and Dorrestein 2011) has been used to visualize the distribution of selected
733 chemicals such as non-ribosomal antifungal peptides produced in interactions between fungi and
734 bacteria (Michelsen et al. 2015). However, isolating natural fungal-bacterial partners is not
735 trivial and there is a need for new techniques, especially for isolating bacteria from fungal
736 surfaces. Some have already been developed and used to isolate bacteria from fungal highways
737 (Simon et al. 2015) or from floating mycelia (Cuong et al. 2011). Another challenge is to grow
738 natural fungal metaorganisms, since maintaining them on standard rich lab-media could interfere
739 with and break up the association, a problem also faced in highly context-specific lichen
740 metaorganisms (Verma and Behera 2015).
741
742 **C.** Physiological processes known from other host-symbiont systems

In this section, we list a few physiological processes known from other host-microbe systems that are also likely to be involved in fungal-bacterial interactions.

Extracellular vesicle trafficking: All organisms can produce extracellular vesicles (Deatherage and Cookson 2012). In fungal pathogens of humans, these exosomes are important in interactions with the host (Rodrigues et al. 2014), whereas in bacteria they play a role in biofilm communication between cells (Remis et al. 2014; Kulp and Kuehn 2010) and interaction with other bacteria (Kulp and Kuehn 2010; Vasilyeva et al. 2013).

Transfer of interfering RNA: Extracellular vesicles have been shown to sometimes carry small RNA (Samuel et al. 2015) or DNA (Kulp and Kuehn 2010), which opens up possibilities for interfering with partner organisms (Nicolás and Ruiz-Vázquez 2013).

Unconventional secretion: Fungi, like all eukaryotes, secrete proteins mainly through the ER-Golgi pathway using N-terminal signal peptides to guide the proteins into the pathway. Proteins without signal peptides can also be secreted through unconventional secretion pathways (Zhang and Schekman 2013). These pathways are important during interaction between host and microorganisms in both plant and animal systems (Ding, Robinson, and Jiang 2014; Öhman et al. 2014) and additionally also involved in the production of extracellular vesicles (Zhang and Schekman 2013).

Priming of responses against pathogens by beneficial organisms: Beneficial bacteria are recognized by similar systems as pathogens and can induce enhanced immune functions against later attacks by pathogens, thus priming the defenses. Such priming responses are a hot topic in both plant and animal systems (Chu and Mazmanian 2013; Conrath 2009; Aranega-Bou et al. 2014; Val et al. 2008) and can be expected to be important for both non-heritable and heritable fungal bacterial interactions.

VII. Closing Remarks

The recent explosion of newly discovered fungal-bacterial interactions suggests that they are more common and important than previously thought. In addition to their significance in ecosystem functioning, many fungal-bacterial associations are central to human health, agriculture, forestry, and bioremediation. While some of these important symbioses are already in the forefront of data gathering and interpretation, many still remain unknown because of the microscopic scale of the interacting partners, the complexity of their communities, and the intricate nature of the relations that connect them. The advent and expansion of new techniques, which allow for exploration and characterization of microbiota in natural and man-made habitats, carries a promise that these obscure systems will soon be discovered and understood at the level achieved for macroorganisms and their interactions. Here, we hope that our discussion will inspire both fungal biologists and prokaryotic microbiologists to develop cross-disciplinary approaches allowing for discovery and characterization of novel links between fungi and bacteria. Until microbiota-specific conceptual tools are established, these explorations could be guided by ecological and evolutionary frameworks that already exist for interspecific interactions among macroorganisms. Collectively, a combination of the omics approaches, genetic experiments, and ecological and evolutionary tools will allow us to expand the knowledge of fungal-bacterial biodiversity and understand the mechanisms underlying these inter-domain interactions.

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Table 1. Mechanisms shared by diverse eukaryotic hosts to select beneficial organisms colonizing host surfaces involved in nutrient uptake.

General for eukaryotic hosts (means)	Host specific (means)
pH reduction by the host (secretion of hydrogen ion)	Secreted antibacterial compounds (production and secretion of secondary metabolites and/or antimicrobial peptides, AMPs)
Host reduction of iron availability (activation of host iron uptake machinery)	Provisioning of beneficial bacteria with specific nutrients not common in other environments. (synthesis and secretion of specific carbon sources)

Figure Captions

Figure 1. Metaorganisms comprise fungal hosts and their various bacterial symbionts.

Figure 2. Evolutionary theory predictions on the role of vertical transmission in the evolution of mutualisms from antagonisms. Hosts are depicted as red ovals; host-positive symbionts are shown as green dots, host-negative symbionts as purple dots. Relative host fitness is reflected by the size of ovals.

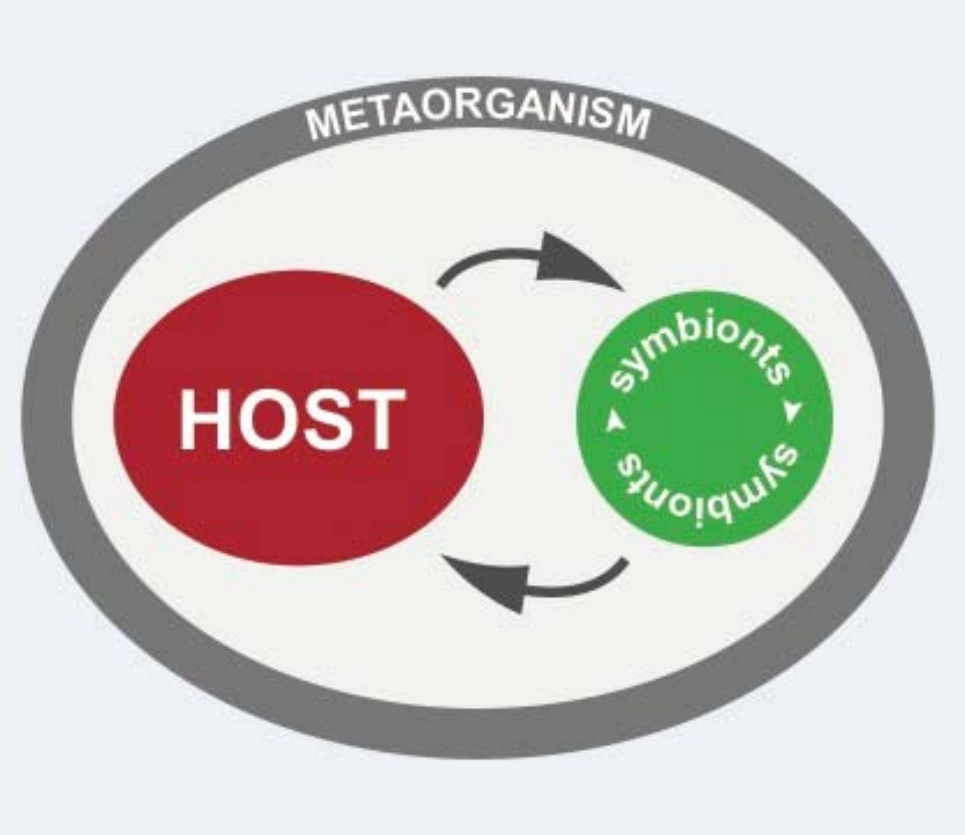
Figure 3. Hypothetical evolutionary trajectories in heritable mutualisms. Hosts are depicted as red ovals; endosymbionts are shown as green dots. Relative host fitness is reflected by the size of ovals. (A) Evolutionary trajectory leading to obligate reciprocal partner dependence. (B) Shifting environmental conditions are expected to arrest an association at the facultative dependence stage. If conditions remain unfavorable for prolonged periods of time, host populations would be expected to completely lose endosymbionts. Modified from Mondo et al. (2012).

Figure 4. Model of plant-fungus-endobacterium interaction (Courtesy of M. Novero). Genome-sequencing results for *Candidatus* Glomeribacter gigasporarum indicate that the bacterium fully depends on the fungal metabolism, including carbon (C), phosphorus (P), and nitrogen (N) metabolism. In contrast, the fungus depends on its green plant host for C uptake only.

Figure 5. Evolutionary theory predictions linking the type of symbiosis with the intra-host relatedness of symbionts and symbiont transmission. Hosts are shown as red ovals. Relative host fitness is reflected by the size of ovals. Endosymbionts are represented by green and purple dots with different shades depicting different genotypes. Modified from Toomer et al. (2015).

1409

1410 Figure 1



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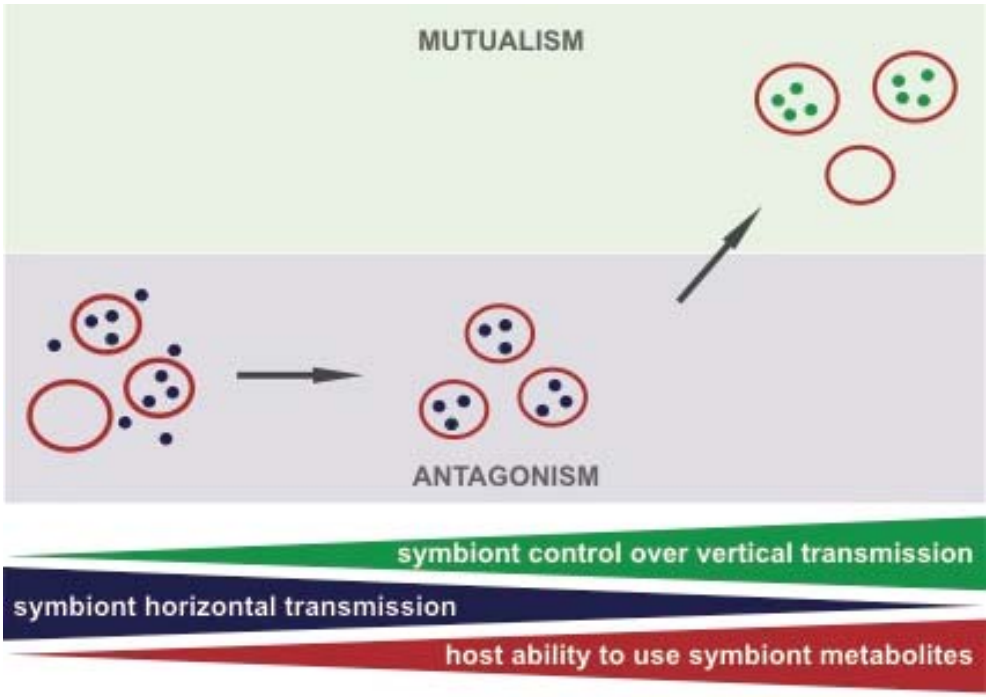
1413 Figure 2



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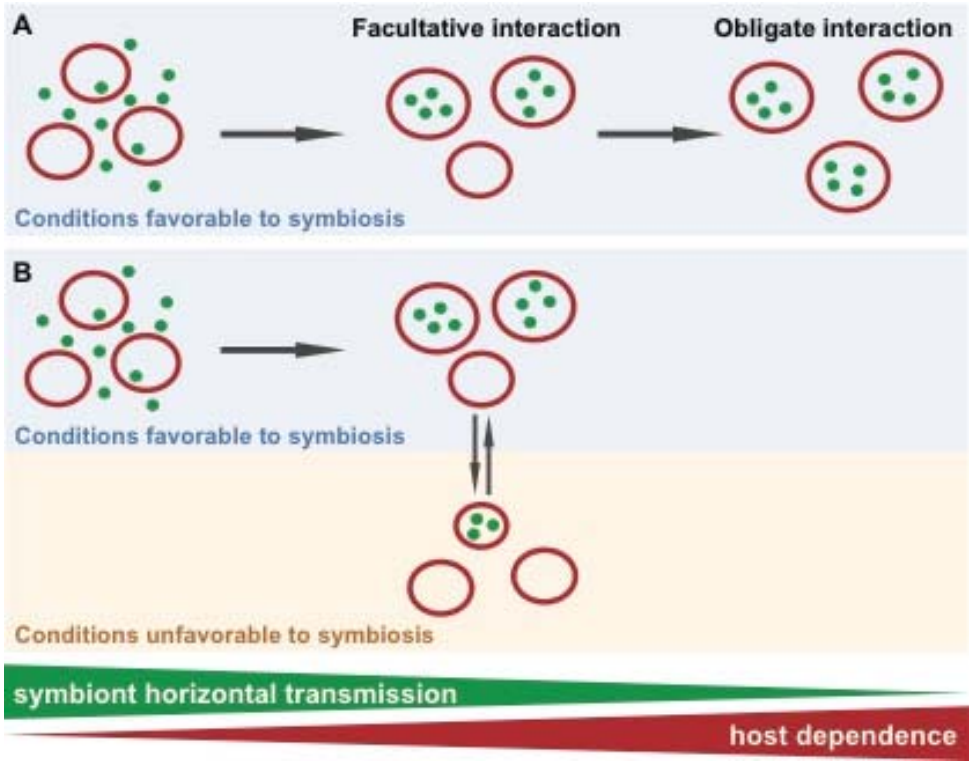
1415

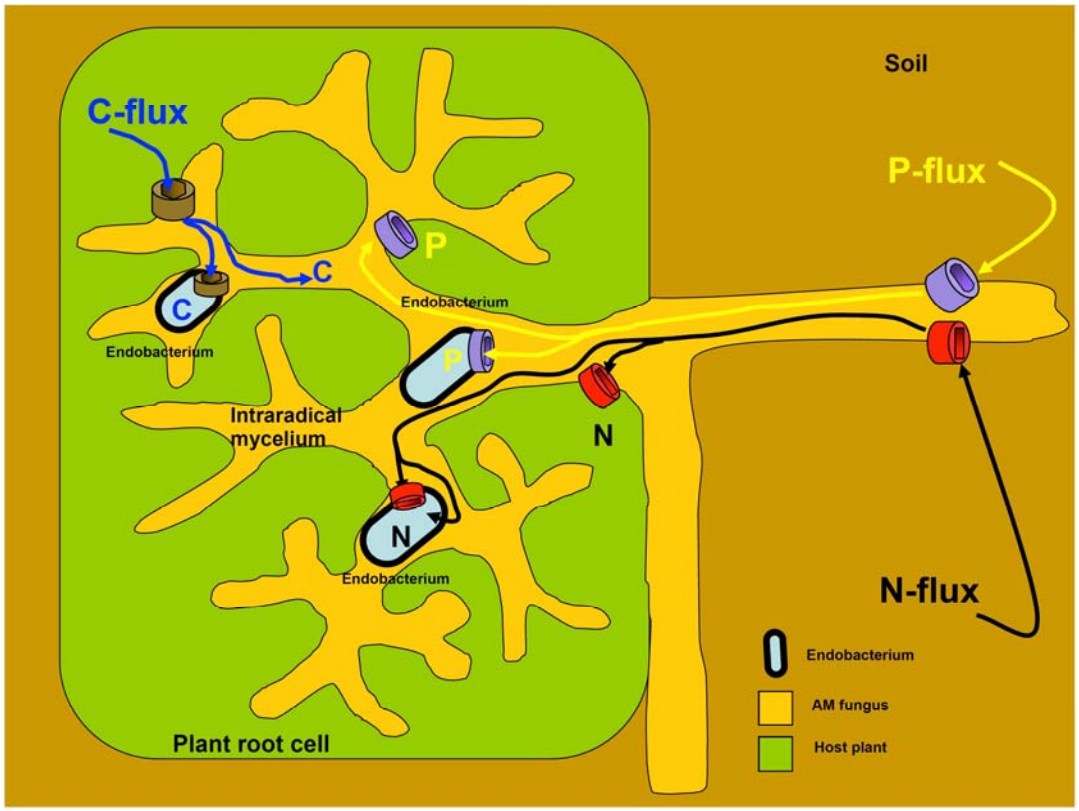
1416 Figure 3



1417

1418

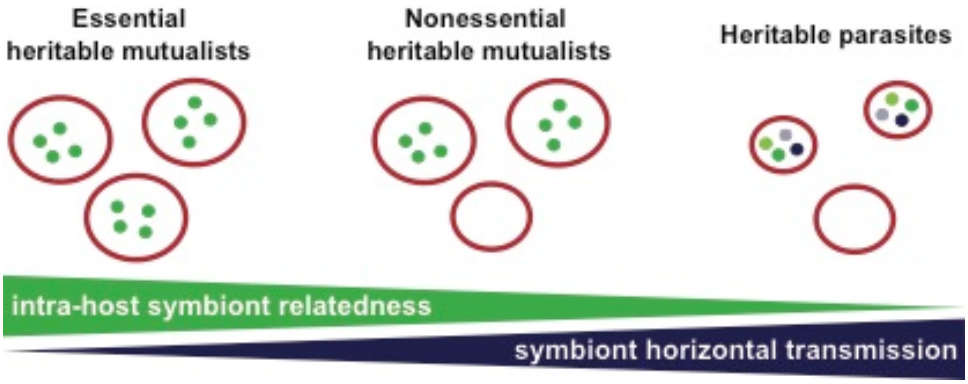
1419 Figure 4



1420

1421

1422 Figure 5



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