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Chapter 39 Ecology and Evolution of Fungal-Bacterial Interactions

This is the author's manuscript
Original Citation:
Availability:
This version is available http://hdl.handle.net/2318/1634115 since 2017-05-15T14:22:26Z
Publisher:
CRC Press Taylor & Francis Group
Published version:
DOI:10.1201/9781315119496-40
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This is the author's final version of the contribution published as:

Olsson, Stefan; Bonfante, Paola; Pawlowska, Teresa E.. Chapter 39 Ecology and Evolution of Fungal-Bacterial Interactions. CRC Press Taylor & Francis Group. 2017. pp: 563-584.

in

The Fungal Community Its Organization and Role in the Ecosystem, Fourth Edition

The publisher's version is available at: http://www.crcnetbase.com/doi/pdf/10.1201/9781315119496-40

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Link to this full text: http://hdl.handle.net/

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1 Ecology and Evolution of Fungal-Bacterial Interactions

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32

I. General Introduction

33 The propensity of fungi to synthesize compounds active against bacteria (Broadbent 1966) and 34 the predilection of bacteria to produce antifungals (Kerr 1999) gave rise to a paradigm that 35 interactions between representatives of these two groups of organisms are of an antagonistic 36 nature. While, indeed, evidence for fungal-bacterial antagonisms is abundant (Espuny Tomas et 37 al. 1982; Leveau and Preston 2008; Susi et al. 2011; Palaniyandi et al. 2013; Pawlowska et al. 38 2012; Pliego et al. 2011), the recent accumulation of newly discovered associations in which 39 fungi cooperate with bacteria (Kobayashi and Crouch 2009; Frey-Klett et al. 2011) indicates that 40 such reciprocally beneficial interactions are more common than previously thought. As 41 functional and mechanistic aspects of many of these interdomain relationships were reviewed in 42 detail elsewhere (Grube and Berg 2009; Kobayashi and Crouch 2009; Peleg, Hogan, and 43 Mylonakis 2010; Frey-Klett et al. 2011; Martin and Schwab 2012; Scherlach, Graupner, and 44 Hertweck 2013), our discussion will focus on factors that contribute to their stability over 45 ecological and evolutionary time. We hope that, by directing attention to this important but

46 currently neglected aspect of fungal-bacterial interactions, we will inspire new directions of

47 research on the biology of these organisms.

48

49 **II. Definitions and Concepts**

50 We use the term **symbiosis** in the de Bary's sense of "the living together of unlike organisms",

- 51 without implications whether this relationship has positive or negative fitness consequences for
- 52 any of the interacting partners (Martin and Schwab 2012). Thus in terms of fitness outcomes, the
- 53 symbiosis can assume the forms of a mutualism (+/+), commensalism (+/0), and antagonism,
 - 3

54	including competition (-/-), amensalism (-/0), parasitism and predation/grazing (-/+) (Lewis
55	1985). We doubt that strictly neutral relationships $(0/0)$ exist among the symbiotic partners.
56	We recognize that practically all biota on the planet are components of stabile assemblages of
57	organisms, referred to as metaorganisms (Bosch and McFall-Ngai 2011). Although not ideal,
58	this term is reasonably well defined and increasingly coming into use (Trinchieri 2014; Biagi et
59	al. 2012). We employ it in our discussions of entities formed in the process and as a
60	consequence of fungal-bacterial interactions (Fig. 1). Thus it is the metaorganism that survives
61	in nature and changes over time due to evolution of its individual constituents, their composition,
62	and the roles in the metaorganism. It is important to note that fungal-bacterial metaorganisms
63	may be, in turn, components of higher-level metaorganisms comprising also plant or animal
64	hosts. We refer to the fungal constituents of the fungal-bacterial metaorganism as the hosts and
65	the bacterial partners as the symbionts. Both hosts and symbionts can be represented by a single
66	species, or they can each comprise a multi-species consortium in which different species interact
67	with each other. In terms of physical interface between the partners, bacterial symbionts can act
68	as endobionts/endosymbionts living intracellularly inside the hyphae, or as
69	ectobionts/epibionts/ectosymbionts/episymbionts associated with the surface of the hyphae or
70	in the close vicinity of the hyphae, often in biofilms consisting of several layers of bacteria held
71	together by a matrix. Metaorganism formation can take several routes. Most known associations
72	of fungi with bacteria are non-heritable , with bacterial symbionts assembled by each generation
73	of the host <i>de-novo</i> from the environment. In contrast, heritable bacterial symbionts are
74	transmitted vertically from the host parent to the next generation of the fungal-bacterial
75	metaorganism. Vertical transmission can be either strict/exclusive, or mixed, <i>i.e.</i> punctuated by
76	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

123	failures. Yet, their evolutionary persistence suggests that certain mechanisms could ensure
124	mutualism stability (Trivers 1971). Several theoretical models have been proposed to explain
125	evolutionary stability of mutualisms. They include: (1) byproduct cooperation (Connor 1986;
126	Sachs et al. 2004), (2) the iterated prisoner's dilemma, IPD, model with the "tit-for-tat"
127	strategy (Axelrod and Hamilton 1981; Doebeli and Knowlton 1998; Sachs et al. 2004), (3)
128	partner-fidelity feedback, PFF (Bull and Rice 1991; Sachs et al. 2004; Weyl et al. 2010), (4)
129	partner choice (Bull and Rice 1991; Noë and Hammerstein 1994; Sachs et al. 2004), and (5)
130	compensatory evolution/addiction (Aanen and Hoekstra 2007). (1) Byproduct cooperation
131	involves interactions in which a focal partner receives a byproduct benefit from a donor and
132	natural selection shapes the focal partner to maximize these benefits by being cooperative toward
133	the donor (Connor 1986; Sachs et al. 2004). (2) The IPD model with the "tit-for-tat" strategy
134	applies to systems in which two partners, who engage in a series of interactions, are able to vary
135	their behavior in each interaction according to a partner's previous action (Axelrod and Hamilton
136	1981; Doebeli and Knowlton 1998; Sachs et al. 2004; Weyl et al. 2010). Cooperation is
137	maintained only when partners reciprocate in kind. Non-cooperative individuals are sanctioned
138	by their partners through termination of cooperation. (3) Like IPD, the PFF model applies to
139	systems in which two partners interact repeatedly. However, in PFF, fitness gains derived from
140	cooperation by one partner feed back to the other partner, thus the partner who fails to cooperate
141	harms its own fitness (Bull and Rice 1991; Sachs et al. 2004; Weyl et al. 2010). (4) Unlike IPD
142	and PFF, the partner choice model involves interactions of a focal individual with multiple
143	trading partners who are reciprocated based on the quality of goods and services offered, with the
144	most cooperative partner receiving the highest compensation (Sachs et al. 2004; Kiers et al.
145	2011). (5) The mechanism of compensatory evolution/addiction is expected to operate in

146	mutualisms that evolved from antagonistic interactions, in host populations exposed initially to a
147	parasitic symbiont (Aanen and Hoekstra 2007). Under parasite pressure, host mutants are
148	favored that compensate for harmful effects of the parasite and thus suffer less damage. Once
149	such compensatory mutations are fixed, they may become deleterious to the host in the absence
150	of the parasite. As a consequence, a host population with such compensatory mutations will
151	become dependent on the presence of the parasite, leading ultimately to a conversion of an
152	antagonistic interaction into a stable mutualism.
153	For the sake of clarity, we divided our discussion of fungal-bacterial symbioses into
154	sections devoted to systems in which partners are assembled <i>de novo</i> in each generation versus
155	associations in which partners are transmitted together from generation to generation and
156	interactions are heritable. We also discussed the role of vertical transmission in evolution of
157	mutualisms from antagonisms. Finally, we suggested tools and future directions for studying
158	fungal-bacterial symbioses.
159	
160	III. Non-Heritable Symbiotic Interactions
161	A. Introduction
162	All basic types of relationships, <i>i.e.</i> mutualisms, commensalisms, and antagonisms, can be found
163	among non-heritable fungal-bacterial symbioses. For some of them detailed knowledge is
164	available, others will be mentioned only briefly. Some bacteria associate directly with fungal
165	hyphae (Baschien et al. 2009; Cuong et al. 2011) and form biofilms on their surfaces (Simon et
166	al. 2015; Pion et al. 2013; Scheublin et al. 2010). These epibionts live in the hyphosphere, the
167	volume around hypha influenced by the hyphal presence (Errore. Riferimento a
168	collegamento ipertestuale non valido.). The bacterial symbionts can be antagonistic, as is

Eliminato: Staněk 1984

169	typical for bacteria used for biocontrol of fungal pathogens (Mela et al. 2011; Cuong et al. 2011;
170	Jochum, Osborne, and Yuen 2006; Mathioni et al. 2013). They can also act as mutualists (Nazir,
171	Tazetdinova, and van Elsas 2014). However, it seems that there is a limited number of
172	fungiphilic bacterial taxa, <i>i.e.</i> taxa adapted to the mycosphere, that are involved in fungal-
173	bacterial symbioses (Lyons et al. 2005; Warmink, Nazir, and van Elsas 2009; Simon et al. 2015;
174	Baschien et al. 2009; Scheublin et al. 2010). Finally, some non-heritable interactions are quite
175	unexpected and thought provoking, like those formed by bacteria-farming fungi (Pion et al.
176	2013), or bacterivorous nematodes and nematophagous fungi (Wang et al. 2014; Hsueh et al.
177	2013).
178	
179	B. Candida albicans-Pseudomonas aeruginosa antagonism
180	Because of their significance to human health, interactions between Candida albicans and

181 *Pseudomonas aeruginosa* attracted a lot of attention, which, in turn, yielded important insights

182 into the molecular mechanisms that underlie the coexistence of these two organisms in the

183 context of human disease (Peleg, Hogan, and Mylonakis 2010). C. albicans is a commensal

184 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

186 mucosal microbiome, such as the use of antibiotics, hormonal imbalance, or diet, can induce

187 non-life threatening C. albicans infections of mucosal surfaces, candidiasis (Scully, el-Kabir, and

188 Samaranayake 1994). In severely ill and immunocompromised individuals, C. albicans can

189 spread into the blood stream causing invasive and often fatal candidaemia (Eggimann, Garbino,

190 and Pittet 2003). C. albicans invasions of host tissues are associated with a morphogenic switch

191 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 \sim 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).

215	Initially, the morphogenic effects of P. aeruginosa-derived C12 compunds on C. albicans
216	were considered to be purely coincidental as these molecules share structural similarity with
217	farnesol. Farnesol is the C12 autoregulatory molecule that controls yeast-to-hypha transition in
218	C. albicans (Hogan, Vik, and Kolter 2004) by modulating cyclic AMP signaling through direct
219	inhibition of the adenylate cyclase activity (Davis-Hanna et al. 2008; Lindsay et al. 2012; Hall et
220	al. 2011) and suppressing filamentation of yeast cells (Hornby et al. 2001). Recent studies
221	revealed that despite structural similarities among the C12 HSLs and their analogs, only
222	3OC12HSL mimics farnesol's activity by interacting with the adenylate cyclase. Another C12
223	compound, dodecanol prevents yeast-to-hypha transition through a different mechanism
224	involving the transcriptional hyphal suppressor Sfl1p (Hall et al. 2011). Interestingly, dodecanol
225	shares structural similarity with a diffusible signal factor of Burkholderia cenocepacia (Hall et
226	al. 2011), which also interferes with C. albicans filamentation (Boon et al. 2008). Like P.
227	aeruginosa, representatives of the B. cepacia complex frequently coexist and interact
228	antagonistically with C. albicans in mixed infections of patients who are immunocompromised
229	and suffer chronic lung disease (Kerr 1994). Notably, however, C. albicans does not seem to
230	respond to C8 HSL, the major quorum-sensing signal produced by <i>B. cepacia</i> (Hogan, Vik, and
231	Kolter 2004; Boon et al. 2008).
232	In addition to autoregulation of fungal morphogenesis, farnesol plays a role in
233	interactions with bacterial antagonists by inhibiting biosynthesis of the P. aerugionosa quinolone
234	signal (PQS) and the PQS-controlled biosynthesis of the pyocyanin siderophore virulence factor
235	(Cugini et al. 2007). Moreover, C. albicans interferes with P. aeruginosa signaling and
236	metabolite production. It can also inhibit virulence of <i>P. aeruginosa</i> in mice by inhibition of
237	bacterial pyochelin and pyoverdine siderophore biosynthesis (Lopez-Medina et al. 2015).

238	While the structural similarity of compounds that suppress yeast-to-hypha transition in <i>C</i> .
239	albicans may suggest ecological fitting, the diversity of the morphogenic mechanisms utilized by
240	C. albicans to respond to these bacterial C12 signal molecules as well as the complex interplay
241	of inhibitory interactions between C. albicans and its bacterial antagonists suggest that these
242	organisms may have been undergoing reciprocal selection within the context of human disease.
243	This process is expected to intensify with the continued increase in the number of patients who
244	require immune system suppression.
245	
246	C. Mycophagy and biological control of fungi by bacteria
247	Fungal hyphae are a potential nutrient and energy source for bacteria. Some bacteria seem to be
248	specialized in feeding on fungi and have been considered mycophagous (Leveau and Preston
249	2008). They have been studied mainly as potential biological control agents aimed toward plant
250	pathogenic fungi (Jochum, Osborne, and Yuen 2006; Yoshida et al. 2012; Selin et al. 2010).
251	These antagonistic bacteria can kill the fungus using a combination of enzymes and antifungal
252	compounds. A well-studied and interesting antifungal compound produced by Lysobacter is
253	HSAF (heat-stabile antifungal factor), a hybrid PKS-NRPS inhibiting the fungal acyl-CoA-
254	dependent ceramide synthase, an enzyme unique to filamentous fungi (Li et al. 2008; Yu et al.
255	2007). This inhibition affects the formation of lipid rafts that are important for proper fungal
256	exocytosis and endocytosis (Li et al. 2006; Alvarez, Douglas, and Konopka 2007).
257	Importantly, most potential biological control organisms have been selected for their
258	ability to produce antifungal compounds on agar plates but it is unclear if they also use the
259	fungus as an energy or carbon source or, indeed, if the same inhibiting compounds are active as
260	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).

265 With the advent of transcriptomics and proteomics, new insights have been gained into 266 these antagonistic of interactions. For example, dual transcriptomic studies of both the fungus 267 and the bacterium challenging each other on agar plates focused on interactions between 268 Aspergillus niger and Collimonas fungivorans (Mela et al. 2011) as well as Rhizoctonia solani 269 and Serratia plymuthica (Gkarmiri et al. 2015; Neupane et al. 2015). In these studies, the 270 partners were not allowed to come into physical contact but could exchange metabolites, and in 271 both cases the portion of the fungal colony that was transcriptionally profiled was the one 272 adjacent to the inhibition zone. Both studies found that the fungi reacted by upregulating defense 273 responses (detoxification, efflux pumps), changes to membrane permeability, and increased 274 oxalate production. In contrast, the only response common in bacteria was the upregulation of 275 genes involved in production of secondary metabolites (Mela et al. 2011; Gkarmiri et al. 2015). 276 The two interactions were in many other ways quite different. The Aspergillus-Collimonas 277 interaction was mainly characterized by a competition for nitrogen (Mela et al. 2011), while the 278 *Rhizoctonia-Serratia* interaction involved a mutual chemical warfare, as both the fungus and the 279 bacteria upregulated transcription of genes responsible for secondary metabolites/toxins and 280 defenses (Gkarmiri et al. 2015; Neupane et al. 2015). 281 Another example of fungal-bacterial antagonistic interactions comes from Magnaporthe

281 Another example of fungal-bacterial antagonistic interactions comes from *Magnaportne* 282 *oryzae* transcriptional responses after direct contact with *Lysobacter enzymogenes*, both a wild
 283 type (WT) strain and a mutant strain deficient in virulence (Mathioni et al. 2013). Four

284	Magnaporthe genes were induced at 3 hours by both WT and mutant bacteria, and two of these
285	were known stress response genes (a laccase and a beta-lactamase). The hypothesis that WT L.
286	enzymogenes is capable of turning off fungal defenses while the mutant could not was used to
287	interpret the data. A total of 463 Magnaporthe genes were down-regulated by WT L.
288	enzymogenes. Of these genes, 100 were up-regulated in interaction with the non-virulent mutant
289	and assumed to be genes involved in the fungal general response/defense against bacteria. These
290	genes are predicted to have roles in carbohydrate metabolism, cellular transport and stress
291	response (Mathioni et al. 2013).
292	The examples discussed above offer glimpses into the vast and complex world of
293	metabolic activities involved in trophic interactions between bacteria and fungi, as we are only
294	starting to uncover and understand these food webs. Clearly, further sustained efforts are needed
295	to identify the players and understand the flows of energy and nutrients that support the
296	communities of fungi and bacteria forming such trophic networks.
296 297	communities of fungi and bacteria forming such trophic networks.
296 297 298	communities of fungi and bacteria forming such trophic networks. D. Fungal predation and dependence on bacteria
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 296 297 298 299 300 301 302 303 304 305 	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-

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 <i>nifH</i> 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

330 farming of bacteria are most likely widespread and underappreciated features of terrestrial

331 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

333 conventional food webs.

334

335 E. Highways carrying hyphae-associated bacteria

336 Fungal hyphae expanding in and through unsaturated soil can spread in a soil volume easier than 337 bacteria, as they can bridge over aerial pores and other hydrophobic regions (Kohlmeier et al. 338 2005). The surfaces of the fungus assimilatory hyphae are hydrophilic and thus the fungal 339 hyphae form hydrophilic tracks through soil. These tracks are referred to as **fungal highways** 340 that the bacteria can follow and are generally regarded as beneficial to both the host and the 341 bacterial symbionts (Kohlmeier et al. 2005). The fungal highways have been studied in relation 342 to dissemination of pollutant-degrading bacteria (Kohlmeier et al. 2005; Furuno et al. 2010). In 343 particular, it has been shown that the fungal hyphae might not just help to spread the bacteria but 344 could also function as conduits of pollutants to bacteria (Banitz et al. 2014; Furuno et al. 2010; 345 Wick et al. 2007). In this respect, substrate is channeled from a source along the hyphae to 346 bacteria that are associated with these hyphae. The fungal host seems to nourish the bacterial 347 symbionts inhabiting and spreading on the highways (Bravo et al. 2013; Nazir et al. 2013). The 348 number of bacterial taxa associating and travelling along the fungal highways is probably a 349 combination of selection for the specific prevalent conditions, available substrates, and also by 350 direct activities of the host, e.g. a consequence of mutualist recognition or absence of parasite 351 recognition. Bacterial motility by flagella as well as other types of motility have been suggested

352	as a common characteristic of bacteria travelling on the fungal highways (Bravo et al. 2013).
353	Among bacterial taxa especially common in the hyphosphere is the genus Burkholderia (Suárez-
354	Moreno et al. 2012). Interestingly, the same genus is also prominent among fungal
355	endosymbionts (see sections below). Fungus-derived oxalate and glycerol have been shown to
356	feed both mutualistic and parasitic bacterial symbionts living and spreading on the fungal
357	highways (Bravo et al. 2013; Nazir et al. 2013). It has also been shown that some bacteria that
358	migrate as "hitchhikers" along fungal highways can only do this if other bacteria have paved the
359	way for them (Warmink et al. 2011). Interestingly, such facilitation does not apply to all bacteria
360	(Warmink et al. 2011). Thus there appear to be three categories of bacteria in relation to
361	movement along fungal hyphae: (1) independent travelers that manage to set up the conditions
362	with the fungal hosts necessary for travel, (2) hitchhikers dependent on the simultaneous
363	presence of the independent travelers, and (3) non-travelers, either not having the properties,
364	such as motility, to move along the fungal highway, or being inhibited by the fungal host and/or
365	the first two types of travelers.
366	The potential importance of fungal highways to the soil bacteria suggests that these
367	interactions may be a common and, until recently, overlooked feature of soil ecosystems.
368	Consequently, the diversity of both fungi that serve as the thoroughfares and their bacterial
369	travelers requires in-depth surveying. The approach of symbiotic network reconstruction appears
370	to be a natural starting point for understanding the rules that govern highway usage. Importantly,
371	while bacterial travelers clearly benefit from highway availability, as it improves their mobility
372	in the soil and may offer a source of nourishment, it is unclear whether fungi receive any benefits
373	from this interaction. Is it a mutualism or an interaction in which the fungal partner remains
374	unaffected or perhaps even harmed?

376 **F.** Mycorrhiza helper bacteria

377 Mycorrhizal fungi form with the roots of terrestrial plants symbiotic associations of distinct 378 morphologies and functions, collectively referred to as mycorrhizas (Smith and Read 2008). In 379 the most common among them, ecto- and arbuscular mycorrhizas, fungi facilitate plant mineral 380 nutrient uptake from the soil in return for photosynthetic carbon. As a consequence, these 381 symbioses are of great significance in both natural and managed ecosystems, with a particular 382 impact on agriculture and forestry. Current observations indicate that mycorrhizas are, in fact, 383 complex multipartner interactions (Bonfante and Anca 2009), due to the presence of bacteria that 384 can be either loosely or tightly associated with mycorrhizal fungi (Jansa, Bukovská, and 385 Gryndler 2013; Bianciotto et al. 2001; Perotto and Bonfante 1997). Garbaye (1994) pioneered 386 the work on these associations with the now widely accepted term **mycorrhiza helper bacteria**, 387 **MHB**, which defines bacteria that help mycorrhizal establishment. Since the time of MHB 388 discovery and thanks to the advent of the omics era, new knowledge and insights have 389 accumulated, with a particular focus on the microbiota present in the rhizosphere and endosphere 390 of poplar (Populus). 391 As a host for both ecto- and arbscular mycorrhizal fungi (AMF), poplar is an excellent 392 model for understanding interactions that govern establishment and functioning of mycorrhizal 393 symbioses, including the role of MHB. For example, the genomes of 21 strains of *Pseudomonas* 394 isolated from the *Populus deltoides* rhizosphere and endosphere have been sequenced (Brown et 395 al. 2012), giving rise to extensive genetic and bioinformatic resources. As a further step, these 396 bacterial isolates were screened for MHB effectiveness expressed as the effects on the Laccaria 397 bicolor S238N growth rate, mycelial architecture, transcriptional changes and symbiosis with

398	three <i>Populus</i> lines, <i>P. tremula</i> \times <i>alba</i> , <i>P. trichocarpa</i> , and <i>P. deltoides</i> . Nineteen of the studied
399	isolates had positive impact on L. bicolor growth (Labbé et al. 2014). Interestingly, one strain
400	promoted high root colonization also in <i>P. deltoides</i> , which is otherwise poorly colonized by <i>L</i> .
401	bicolor. In this context, the genome of a MHB isolate of Pseudomonas fluorescens BBc6R8 will
402	be of great advantage in identifying the helper traits (Deveau et al. 2014).
403	Prokaryotes are associated not only with the extraradical hyphae of mycorrhizal fungi,
404	but also with ectomycorrhizal roots and sporocarps, <i>i.e.</i> , the fruiting bodies formed by
405	ectomycorrhizal ascomycetes and basidiomycetes, suggesting that they may accompany
406	mycorrhizal fungi during the various steps of their life cycle. Because of their economic
407	significance, Tuber sporocarps have become a model to understand a role that truffle-associated
408	bacteria play in several still poorly understood aspects of truffle development, from fruiting body
409	formation to aroma production. Similarly, the appearance of the "brûlé", an area devoid of
410	vegetation around the <i>Tuber</i> host plants and where the fruiting bodies of <i>T. melanosporum</i> are
411	usually collected, is a feature with a clear ecological impact but largely unknown causes. For
412	example, the examination of direct fungal-fungal interactions (Napoli et al. 2010), together with
413	DGGE and DNA microarray analyses of 16S rRNA gene fragments (Mello et al. 2013), revealed
414	that the bacteria and archaeal communities strongly differ between the inside versus outside of
415	the brûlé area. The groups that were most severely affected by the black truffle included
416	Firmicutes, several genera of Actinobacteria, and a few Cyanobacteria. One of the mechanisms
417	responsible for this pattern could be the capacity of truffles to release volatile organic
418	compounds (Splivallo et al. 2011). Intriguingly, Splivallo et al. (2015) found that sulphur-
419	containing volatiles, such as thiophene derivatives characteristic of <i>T. borchii</i> fruiting bodies, are
420	products of the bacteria-mediated biotransformation of non-volatile precursor(s) into volatile

421 compounds. Moreover, the α - and β -proteobacteria-dominated community of *T. borchii* was able 422 to produce thiophene volatiles from *T. borchii* fruiting body extract, irrespective of their isolation 423 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.

428

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

Both plant and animal epithelial surfaces coming in contact with bacteria share a similar problem

432 in that they should actively select for beneficial/commensal bacteria and discourage the

433 colonization by antagonists (McFrederick et al. 2012; Artis 2008; Ausubel 2005; Zamioudis and

434 Pieterse 2012). Innate immunity recognition of bacterial cues as MAMPs (microbial associated

435 molecular patterns) plays a key role in this selection in both plants and animals (Artis 2008;

436 Nürnberger et al. 2004). However, the immune reaction is balanced so as not to kill eventual

437 beneficial bacteria, as is done in tissues not normally colonized by bacteria (Artis 2008;

438 Zamioudis and Pieterse 2012). Fungal hyphae growing in most natural environments face a

439 similar need to promote the beneficial and inhibit the antagonistic microbes. Fungal reactions to

440 a bacterial MAMP have been demonstrated (Xu et al. 2008), the existence of innate immunity

441 type recognition has been suggested (Paoletti and Saupe 2009; Paoletti, Saupe, and Clavé 2007),

442 and recently transcriptomic innate immunity type responses have been found in fungi (Ipcho et

443 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.
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490	(Yamamura 1993) (Fig. 2). If the increase in the rate of symbiont vertical transmission is
491	accompanied by the development of host abilities to complement its metabolism using symbiont
492	metabolites, a byproduct mutualism is expected to evolve (Yamamura 1993).
493	While the model that explains the evolution of mutualisms from antagonisms through
494	changes in the rates of symbiont transmission is rather straightforward (Yamamura 1993), the
495	actual mechanisms that permit symbiont vertical transmission remain elusive as nearly all known
496	heritable endosymbionts are uncultivable (Moran, McCutcheon, and Nakabachi 2008) and many
497	hosts are unable to survive without their endobacteria. In this context, the rice seedling blight
498	fungus Rhizopus microsporus and its endosymbiont Burkholderia rhizoxinica offer an
499	unprecedented opportunity to understand the evolution of mutualisms from antagonisms
500	(Partida-Martinez and Hertweck 2005; Lackner and Hertweck 2011). In this system, the
501	endobacteria reside directly within the fungal cytoplasm (Partida-Martinez et al. 2007). Their
502	elimination with antibiotics abolishes fungal ability to form asexual sporangia and
503	sporangiospores (Partida-Martinez et al. 2007), suggesting that endobacteria not only gained
504	control of their own transmission rate but also of the reproductive success of the fungus, a
505	pattern consistent with the compensatory evolution/addiction model of mutualism evolution
506	(Aanen and Hoekstra 2007). In addition to controlling the rate of own vertical transmission by
507	rendering fungal reproduction dependent on their presence, the endobacteria produce a macrolide
508	metabolite that is processed by the host to form a highly potent antimitotic toxin called rhizoxin
509	(Scherlach et al. 2012). The toxin is active in rice seedlings, where it causes the blight disease
510	(Lackner, Partida-Martinez, and Hertweck 2009). In addition, rhizoxin is believed to facilitate
511	competitive interactions of the Rhizopus host with fungi that are sensitive to it. Such positive
512	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 <i>Rhizopus</i> for entering a byproduct mutualism with the <i>Burkholderia</i>
518	endobacteria.
519	The 3.75 Mb genome of <i>B. rhizoxinica</i> 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 <i>Candidatus</i>
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 <i>B. rhizoxinica</i> 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).

536 Even though some of the features displayed by the *Rhizopus-Burkholderia* symbiosis are 537 typical for a mutualism, the *Burkholderia* endobacteria appear to be facultative endosymbionts, 538 capable of living both inside and outside eukaryotic cells, a lifestyle similar to that of pathogenic 539 Legionella, Salmonella, or Bartonella. This duality, combined with the ease of experimental 540 manipulation, propelled the Rhizopus-Burkholderia symbiosis to become a model for studying 541 the evolution of heritable symbioses. In particular, addressing questions concerning its 542 evolutionary origins, whether it started with the partners interacting as antagonists (Fig. 2), and 543 whether it has already achieved evolutionary stability (Fig. 3) will be a source of rich insights not 544 only into the genetic mechanisms of symbiont vertical transmission but also into other facets of 545 partner coevolution. 546 547 V. Heritable Symbiotic Interactions 548 **A.** Introduction 549 As discussed in the preceding sections, symbiont vertical transmission is a principal factor

550 contributing to both the establishment and stability of mutualisms. Importantly, vertical 551 transmission is not exclusive to mutualisms; it can also occur in antagonistic interactions. 552 Vertical transmission can be strict or mixed. In strict vertical transmission symbionts are 553 transferred from a parent exclusively to offspring. In mixed transmission, in addition to being 554 passaged between generations, symbionts move horizontally between members of the same 555 generation. Symbioses with strict vertical transmission are characterized by congruity of partner 556 phylogenetic histories, consistent with partner codiversification (Page 2003). In symbioses with 557 mixed transmission, the extent of horizontal transmission determines the degree of incongruity 558 between partner phylogenies. Interestingly, strict vertical transmission of symbionts tends to be

associated with reciprocally obligate partner dependence, whereas mixed transmission is found
in associations in which either one or both partners are facultatively dependent on the symbiosis
(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

and Arnold 2010). Due to the lack of sufficient data from other systems, our discussion in the

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

605	for nutrients and energy (Fig. 4). Interestingly, the whole operon for biosynthesis of vitamin B12
606	is present in the Glomeribacter genome, but it is not clear whether this might represent any
607	benefit for the fungus. In contrast to animals, which use B12-dependent enzymes for methionine
608	synthesis and methylmalonate metabolism, fungi and land plants rely on B12-independent
609	enzymes for these pathways (Young, Comas, and de Carvalho 2015). Consistent with this
610	expectation, the genome of a model AMF, Rhizophagus irregularis, encodes B12-independent
611	enzymes (Tisserant et al. 2013).
612	While the significance of <i>Glomeribacter</i> to the AMF hosts could not be gleaned from its
613	genomic sequence, the availability of a stable endosymbiont-free AMF Gigaspora margarita
614	BEG34 line, designated as B(-), allowed for direct comparisons with the line containing the
615	endobacterium, B(+). These comparisons revealed several differences, both phenotypic (Lumini
616	et al. 2007) and transcriptional (Salvioli et al. 2016), that speak to the role of Glomeribacter in
617	the AMF host. For example, the B(-) AMF line was able to colonize its plant host but was
618	impaired in mycelial growth and spore production compared to the B(+) line (Lumini et al.
619	2007). Moreover, benefits of the endosymbiont presence appeared to extend to the plant host, as
620	the phosphate measurements in Lotus japonicus plants revealed a statistically higher phosphate
621	quantity in the symbiosis established by the $B(+)$ versus the $B(-)$ AMF line (Salvioli et al. 2016).
622	In turn, the transcriptome analysis showed that the endobacterium had a stronger effect on the
623	pre-symbiotic phase of the fungus, supporting earlier phenotypic observations that

624 *Glomeribacter* promotes germ tube extension in the AMF host (Lumini et al. 2007; Salvioli et al.

- 625 2016). Coupling of transcriptomics with physiological and cell biology approaches
- 626 demonstrated that the bacterium increases the AMF sporulation success, raises the AMF
- 627 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

vulnerable to genomic degeneration and extinction (McCutcheon and Moran 2012). Yet, co-

697	divergence patterns between MRE and the two fungal lineages in which MRE occur, AMF and
698	the Endogone lineage of Mucoromycotina, suggest that the AMF-MRE association may predate
699	the divergence between these two lineages and thus be as old or older than the Gigasporaceae-
700	Glomeribacter symbiosis (Desirò et al. 2015; Toomer et al. 2015). It has been postulated that the
701	key factors that prevent MRE from extinction are the mechanisms responsible for genome
702	plasticity in MRE, including the recombination machinery and mobile genetic elements, (Naito,
703	Morton, and Pawlowska 2015; Naito and Pawlowska 2016). Despite these advances, MRE
704	remain an elusive group of endobacteria. Not only their role in the AMF host biology but also
705	the mechanisms of putative horizontal transmission require experimental evaluation.
706	
707	VI. Future Developments
708	A. Introduction
709	The establishment and outcomes of the fungal bacterial interactions are most probably a result of
710	chemical communication where a compound from one partner elicits a response with another
711	compound from the other partner (Baruch et al. 2014; Piispanen and Hogan 2008; Xu et al. 2008;

712 Badri et al. 2009; Nazir et al. 2010; Schroeckh et al. 2009; Sengupta, Chattopadhyay, and

713 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
- 717 modern omics, these ping-pong events could be studied using transcriptomics (Mela et al. 2011;
- 718 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
 - 32

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

- 743 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.
- 745 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).
- 750 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
- 752 interfering with partner organisms (Nicolás and Ruiz-Vázquez 2013).
- 753 Unconventional secretion: Fungi, like all eukaryotes, secrete proteins mainly through the ER-
- 754 Golgi pathway using N-terminal signal peptides to guide the proteins into the pathway. Proteins
- vithout 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.
- 758 2014) and additionally also involved in the production of extracellular vesicles (Zhang and
- 759 Schekman 2013).
- 760 **Priming of responses against pathogens by beneficial organisms:** Beneficial bacteria are
- 761 recognized by similar systems as pathogens and can induce enhanced immune functions against
- 762 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.
- 764 2014; Val et al. 2008) and can be expected to be important for both non-heritable and heritable
- 765 fungal bacterial interactions.
| 767 | VII. Closing Remarks |
|-----|--|
| 768 | The recent explosion of newly discovered fungal-bacterial interactions suggests that they are |
| 769 | more common and important than previously thought. In addition to their significance in |
| 770 | ecosystem functioning, many fungal-bacterial associations are central to human health, |
| 771 | agriculture, forestry, and bioremediation. While some of these important symbioses are already |
| 772 | in the forefront of data gathering and interpretation, many still remain unknown because of the |
| 773 | microscopic scale of the interacting partners, the complexity of their communities, and the |
| 774 | intricate nature of the relations that connect them. The advent and expansion of new techniques, |
| 775 | which allow for exploration and characterization of microbiota in natural and man-made habitats, |
| 776 | carries a promise that these obscure systems will soon be discovered and understood at the level |
| 777 | achieved for macroorganisms and their interactions. Here, we hope that our discussion will |
| 778 | inspire both fungal biologists and prokaryotic microbiologists to develop cross-disciplinary |
| 779 | approaches allowing for discovery and characterization of novel links between fungi and |
| 780 | bacteria. Until microbiota-specific conceptual tools are established, these explorations could be |
| 781 | guided by ecological and evolutionary frameworks that already exist for interspecific interactions |
| 782 | among macroorganisms. Collectively, a combination of the omics approaches, genetic |
| 783 | experiments, and ecological and evolutionary tools will allow us to expand the knowledge of |
| 784 | fungal-bacterial biodiversity and understand the mechanisms underlying these inter-domain |
| 785 | interactions. |
| 786 | |

787 Acknowledgements

788 We thank Olga Lastovetsky for comments on the manuscript. This work was supported by the

- 789 National Science Foundation grant IOS-1261004 to TEP and the Torino University 60% grant to
- 790 PB.

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- 1379 1380
 Table 1. Mechanisms shared by diverse eukaryotic hosts to select beneficial organisms
- 1381 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)

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- 1	- 5	82	

1383 Figure Captions

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

1386 Figure 2. Evolutionary theory predictions on the role of vertical transmission in the 1387 evolution of mutualisms from antagonisms. Hosts are depicted as red ovals; host-positive 1388 symbionts are shown as green dots, host-negative symbionts as purple dots. Relative host fitness 1389 is reflected by the size of ovals. 1390 1391 Figure 3. Hypothetical evolutionary trajectories in heritable mutualisms. Hosts are 1392 depicted as red ovals; endosymbionts are shown as green dots. Relative host fitness is reflected 1393 by the size of ovals. (A) Evolutionary trajectory leading to obligate reciprocal partner 1394 dependence. (B) Shifting environmental conditions are expected to arrest an association at the 1395 facultative dependence stage. If conditions remain unfavorable for prolonged periods of time, 1396 host populations would be expected to completely lose endosymbionts. Modified from Mondo et

1397 al. (2012).

1398

1399 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

1403 only.

1404

1405 Figure 5. Evolutionary theory predictions linking the type of symbiosis with the intra-host

1406 relatedness of symbionts and symbiont transmission. Hosts are shown as red ovals. Relative

- 1407 host fitness is reflected by the size of ovals. Endosymbionts are represented by green and purple
- 1408 dots with different shades depicting different genotypes. Modified from Toomer et al. (2015).

1410 Figure 1



1413 Figure 2



1416 Figure 3





1419 Figure 4


1422 Figure 5

