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Abstract

Heritable symbioses, in which endosymbiotic bacteria (EB) are transmitted vertically between host generations, are an important source of evolutionary novelties. A primary example of such symbioses is the eukaryotic cell with its EB-derived organelles. Recent discoveries suggest that endosymbiosis-related innovations can be also found in associations formed by early divergent fungi in the phylum Mucoromycota with heritable EB from two classes, Betaproteobacteria and Mollicutes. These symbioses exemplify novel types of host-symbiont interactions. Studies of these partnerships fuel theoretical models describing mechanisms that stabilize heritable symbioses, control the rate of molecular evolution, and enable the establishment of mutualisms. Lastly, by altering host phenotypes and metabolism, these associations represent an important instrument for probing the basic biology of the Mucoromycota hosts, which remain one of the least explored filamentous fungi.

1. Introduction

Fungi are increasingly appreciated for their ability to form intimate associations with bacteria (31, 89). Among them, the symbioses of early divergent fungi in the phylum Mucoromycota with an array of heritable endosymbiotic bacteria (EB) from two classes, Betaproteobacteria and Mollicutes, stand out as the most highly co-evolved and ancient. The clade of Mucoromycota includes three subphyla, Mucoromycotina, Mortierellomycotina, and Glomeromycotina (115). Most Mucoromycota engage in plant-related lifestyles of decomposers of plant debris, plant mutualists, and plant pathogens (115). Interactions with animals are uncommon in this group of fungi.

Partnerships with bacteria formed by Mucoromycota have diverse fitness outcomes, involve transfer of various goods and services, and represent a range of degrees of co-evolution. In this review, we will focus on four very distinct symbioses partnering arbuscular mycorrhizal fungi (AMF, subphylum Glomeromycotina) with '*Candidatus* Glomeribacter gigasporarum' (*Ca*Gg, Betaproteobacteria, **Fig 1**) and '*Candidatus* Moeniiplasma glomeromycotorum' (*Ca*Mg, Mollicutes, **Fig 2**) as well as on associations of *Rhizopus microsporus* (*Rm*, subphylum Mucoromycotina) with *Burkholderia* EB (Betaproteobacteria, **Fig 1**), and *Mortierella elongata* (*Me*, subphylum Mortierellomycotina) with *Mycoavidus cysteinexigens* (*Mc,* Betaproteobacteria, **Fig 1**). Despite their marked differences, these Mucoromycota-EB associations provide

67 important insights into the host-symbiont biology. Studies of these symbioses inform
68 evolutionary models describing the mechanisms that stabilize heritable symbioses, control the
69 rate of molecular evolution, and lead to the establishment of mutualisms. In addition, by altering
70 host phenotypes and metabolism, these partnerships are a valuable source of information about
71 the biology of Mucoromycota, which remain one of the least explored groups of filamentous
72 fungi.

73 Heritable symbioses in which EB are transmitted from one host generation to the next can
74 range from antagonisms to mutualisms. Importantly, strictly vertically transmitted symbionts
75 that lower host fitness are unlikely to persist in a host population (28, 60). Evolutionary stability
76 of such antagonistic symbioses requires that, in addition to passaging from parents to offspring,
77 symbionts engage in horizontal transmission between hosts (28, 60). Alternatively, harmful
78 symbionts can be maintained stably if they deliver occasional benefits to the host, forming a
79 conditional mutualism (40, 61, 62, 105).

80 Mutualisms are reciprocal exploitations that nonetheless provide net benefits to each
81 partner (42). This definition emphasizes an inherent vulnerability of mutualisms to instabilities
82 and breakdowns, which stem from conflicting interests of the interacting partners. Vertical
83 transmission is a powerful mechanism that stabilizes mutualisms over evolutionary time (1, 4,
84 18, 21, 27, 104, 130). This stabilizing role is related to the fact that heritability of symbionts
85 aligns partner reproductive interests and facilitates reciprocal selection. While coupling of
86 reproductive efforts maximizes fitness of the partners, it does not eliminate conflicts among the
87 symbionts. Such conflicts are a potential source of instabilities in heritable mutualisms. They
88 intensify when symbiont populations are genetically diverse due to symbiont mixing, which can
89 lead to the emergence of rivaling strategies for the utilization of host resources (30).

90 In established mutualisms, several tactics are possible to control symbiont mixing,
91 including uniparental inheritance of symbionts (13), transmission of only a fraction of parental
92 symbionts to each offspring (29), and separation of an intrahost symbiont population into a
93 reproductive germline and a non-reproductive somatic lineage (29). Host control over symbiont
94 mixing evolved independently multiple times in various symbiotic systems, including eukaryotic
95 cells and their organelles (13) as well as nutritional symbioses of insects that rely on EB for
96 essential metabolites, such amino acids and vitamins (67, 74). While beneficial to the host, long-
97 term evolutionary consequences of suppressed symbiont mixing can be detrimental to the

98 symbionts and the symbiosis as a whole. Symbiont population subdivisions, transmission
99 bottlenecks, and clonality reduce the effective size of a symbiont population and magnify the
100 impact of genetic drift relative to natural selection (90). As a consequence, symbiont populations
101 become vulnerable to accumulation of slightly deleterious (88) and eventual extinction (78). In
102 heritable EB, this process is associated with genomic decay and reduction of the genome size (7,
103 67, 74, 84). Such degenerative genome evolution has been observed empirically in free-living
104 bacteria evolving under conditions of a small effective population size (84), and inferred from
105 molecular evolution patterns in multiple heritable EB that provision insects with essential
106 metabolites (7, 67, 74). Another important consequence of degenerative evolution in heritable
107 EB is acceleration of the molecular evolution rate compared to free-living relatives (73, 87).
108 Remarkably, most of the Mucoromycota-EB symbioses are ancient (15, 72, 121, 124).
109 Two are mutualisms (AMF-*Ca*Gg and *Rm-Burkholderia*), one is an antagonism (*Me-Mc*), and
110 one remains unresolved in terms of partner fitness outcomes (AMF-*Ca*Mg). As a consequence,
111 Mucoromycota-EB associations exemplify diverse mechanisms that control evolutionary
112 stability and longevity in symbioses with vertically transmitted EB. Moreover, with the
113 exception of *Burkholderia* EB, symbionts of Mucoromycota appear to evolve faster than their
114 free-living relatives (20, 81), and thus offer insights into how molecular rate acceleration is
115 achieved in EB with different lifestyles. In addition, these symbioses allow for exploring
116 theoretical predictions that specify conditions necessary for mutualisms to arise. Many such
117 predictions have not been tested rigorously because very few heritable partnerships outside
118 Mucoromycota are amenable to experimental manipulation.
119 In this review, we summarize key features of Mucoromycota-EB partnerships, use
120 molecular evolution patterns apparent in these symbioses to speculate about uncertainties
121 surrounding some of their aspects, describe how studies of the Mucoromycota-EB associations
122 inform and validate theoretical models of symbiosis evolution, and detail how they can be used
123 to generate specific insights into the facets of host biology that historically have been recalcitrant
124 to investigation. In the process, we highlight future research directions.
125

126 ## 2. Host-symbiont biology and symbiosis stability

127 ### 2.1. AMF-*Ca*Gg mutualism

128 *Ca*Gg is a betaproteobacterium (**Fig 1**) and a mutualist of AMF from the family Gigasporaceae
129 (9, 64, 72). AMF are obligate biotrophs that colonize roots of most terrestrial plants and
130 facilitate plant uptake of mineral nutrients from the soil (114) in exchange for photosynthesis-
131 derived monosaccharides (41) and fatty acids (17, 44, 48, 63) coming from the plant. The
132 association that AMF form with plants, arbuscular mycorrhiza, dates back to the Early Devonian,
133 400 MYA (97), and is one of the oldest mutualisms on the planet. AMF are increasingly
134 recognized in agronomy as sustainable biofertilizers of the future (127).
135 *Ca*Gg is vertically transmitted through AMF generations (10) and shows variable
136 distribution across host populations, with some AMF individuals harboring the EB and some
137 being *Ca*Gg-free (12, 72). This pattern suggests that *Ca*Gg is a nonessential partner of AMF.
138 Serial sub-culturing of AMF can lead to elimination of *Ca*Gg under laboratory conditions (64).
139 For AMF, phenotypic consequences of *Ca*Gg loss include reduced elongation and branching of
140 pre-symbiotic hyphae that emerge from spores in the presence of plant roots (64) (**Fig 3**). At the
141 subcellular level, the absence of *Ca*Gg from pre-symbiotic hosts is accompanied by a decline in
142 the volume of lipid droplets present in fungal cells (64). Without *Ca*Gg, spore fatty acids
143 become less abundant, with particular depletion of palmitic acid (106). Pre-symbiotic AMF are
144 unable to synthesize palmitate (123) because they lack genes encoding the fatty acid synthase
145 enzyme complex (118, 129). Consequently, the efficiency of how spore energy reserves are
146 utilized is important for the AMF ability to associate with a plant host. In fungi cured of *Ca*Gg,
147 reductions in lipid droplet volume and fatty acid abundance are accompanied by elevated
148 expression of genes and proteins involved in beta-oxidation of fatty acids and the pentose
149 phosphate pathway, suggesting a shift towards pathways that provide reducing power (126). In
150 contrast, pre-symbiotic fungi harboring *Ca*Gg acquire their reducing power due to elevated
151 mitochondrial oxidative phosphorylation and ATP biosynthesis (107, 126). These increases are
152 associated with respiration rates 50% higher than in the cured fungi (126). Overall, *Ca*Gg
153 appears to interact with AMF energy metabolism in ways that mobilize ATP and fuel pre-
154 symbiotic growth. Interestingly, similar effects are caused by strigolactones, plant hormones that
155 AMF perceive and respond to by enhancing hyphal branching, proliferation of mitochondria and
156 increasing respiration (8, 54). Remarkably, the strigolactone treatment also induces a
157 proliferation of *Ca*Gg cells (3), which suggests that the fungal mitochondrion might be the

158 primary target of both *Ca*Gg and plant strigolactones. However, the proximate mechanism of
159 how *Ca*Gg regulates pre-symbiotic activities of AMF remains elusive.
160 As we discussed earlier, in heritable EB that provision insects with essential metabolites,
161 genes in all functional categories are vulnerable to accumulation of slightly deleterious mutations
162 and decay (7, 67, 74). However, the symbiont genes responsible for essential services to the
163 host, such as those needed for the biosynthesis of amino acids (112) or vitamins (2), maintain
164 their functionality due to host-level selection (19). These observations suggest that clues
165 concerning *Ca*Gg factors that interact with AMF metabolism might be gleaned from the *Ca*Gg
166 genomic data. With sizes ranging from 1.34 Mb to 2.36 Mb (36, 71), the genomes of *Ca*Gg are
167 substantially streamlined compared to their free-living *Burkholderia* relatives (131). However,
168 there are reasons to suspect that the mechanisms of genome contraction in *Ca*Gg are different
169 from those that govern degenerative genome reduction in heritable EB of insects. In particular,
170 *Ca*Gg rate of mutation accumulation of 2.03×10^{-9} substitutions per site per year (71) is
171 comparable to that of free-living bacteria, and much lower than 2.2×10^{-7} substitutions per site
172 per year estimated in *Buchnera aphidicola, Ba* (76). *Ba* is an essential mutualist that provisions
173 phloem-feeding aphids with amino acids missing from their sugar-rich diet, and a model for
174 understanding degenerative genome evolution in heritable EB (77, 112, 117, 128). Importantly,
175 unlike heritable essential mutualists of insects, *Ca*Gg shows evidence of rare recombination and
176 host switching/horizontal transmission (71, 72). This pattern is consistent with a relatively large
177 effective size of the *Ca*Gg population estimated at 1.44×10^{8} (71) and larger than 1.0×10^{7} in *Ba*
178 (34). Accordingly, forces of natural selection are expected to operate in the *Ca*Gg population,
179 and in fact, *Ca*Gg appears to be as effective at purging slightly deleterious mutations as free-
180 living bacteria (71). As a consequence, only the genes encoding biosynthesis of costly
181 metabolites available to *Ca*Gg from the host are expected to be lost from *Ca*Gg genomes.
182 Consistent with this prediction, *Ca*Gg appears to rely on host-derived arginine as its energy
183 source (36). Conversely, EB retains the capacity for the energetically expensive and complex
184 biosynthesis of vitamin B_{12} (36), which is a cofactor essential to some bacteria and humans but
185 has no apparent role in the metabolism of fungi (99, 120). Consequently, the vitamin B_{12}
186 biosynthetic pathway must be preserved by *Ca*Gg for its own benefit. These patterns suggest
187 that identifying genomic clues to how *Ca*Gg reprograms the energy metabolism of its fungal host
188 may not be as simple as in heritable EB with degenerate genomes.

189 *Ca*Gg is transmitted uniparentally, along clonal lineages of its AMF hosts. AMF show
190 no direct evidence of sexual mating and rely on large multinucleate spores for asexual
191 proliferation. Intrahost populations of *Ca*Gg are genetically uniform (72). Such genetic
192 homogeneity could be attributed to a rate of mutation accumulation in *Ca*Gg that is comparable
193 to that of free-living bacteria (71). This low mutation rate (71) and a relatively large effective
194 population size in *Ca*Gg (71) are also likely to be responsible for the extraordinary evolutionary
195 longevity of the AMF-*Ca*Gg symbiosis, which dates back to the Early Devonian (72).
196 What remains uncertain are the forces that allow *Ca*Gg to maintain a relatively large
197 population size. It is possible that the ultimate cause is related to the nature of *Ca*Gg association
198 with AMF. *Ca*Gg services are not essential to AMF, or, in other words, AMF are only
199 facultatively reliant on *Ca*Gg (64, 72). Such reliance suggests that fitness benefits of carrying
200 EB vary depending on specific conditions, with certain environments favoring EB presence and
201 others selecting against it (101, 102). A variable selective landscape is expected to support
202 retention of genetic competence for horizontal transmission and recombination (85), which are
203 present in *Ca*Gg (71, 72). However, the specific environmental factors responsible for AMF
204 facultative rather than obligate dependence on *Ca*Gg are unknown. It could be speculated that
205 these factors are related to conditions affecting pre-symbiotic activities of obligately biotrophic
206 AMF, such as the number of spore germination attempts and the extent of hyphal proliferation.
207

208 **2.2. *Rm-Burkholderia* mutualism**

209 *Rm,* like most other Mucoromycotina, is a saprotroph that also can act as an opportunistic
210 pathogen of plants and humans (93, 108). While multiple *Burkholderia* EB species have been
211 found in different isolates of this fungus, such as *Burkholderia rhizoxinica, Br* (51, 68, 95, 96,
212 113, 125), *Burkholderia endofungorum* (94) and *Burkholderia* sp. (55, 70) (**Fig 1**), no
213 *Burkholderia* EB have been found in other Mucoromycotina (111). Moreover, even within *Rm*
214 some strains do not harbor these EB (55, 93).
215 The *Rm-Burkholderia* mutualism has become a model for understanding fungal-bacterial
216 symbioses because it can be manipulated experimentally, hosts can be cured of symbionts, and
217 partners separated and reassembled back into a functional symbiosis (51, 55, 68, 70, 95). This
218 versatility is related to the genomic makeup of *Burkholderia* EB. The 3.75 Mb genome of *Br*
219 (52) supports functional capabilities important for *Burkholderia* EB persistence outside the host

220 cellular environment and host recolonization as well as endosymbiotic lifestyle and vertical
221 transmission (51, 68, 95). Recolonization of the fungal mycelium is possible due to the activity
222 of *Burkholderia* secretion systems. These systems include the Type II Secretion System, which
223 translocates fungal cell wall-degrading enzymes chitinase and chitosinase (68) as well as the
224 Type III Secretion System (51), which delivers effectors for host manipulation directly into the
225 host cytoplasm (22). The establishment of symbiosis is associated with alterations of the *Rm*
226 lipid metabolism (55). Host lipids are also important for the maintenance of the symbiosis, as
227 they likely provide substrates for *Burkholderia* energy metabolism (52, 53) (**Fig 3**).
228 Nearly 10% of the *Br* genome is comprised of secondary metabolite gene clusters (52,
229 53). Secondary metabolites are low molecular weight compounds with potent physiological and
230 antimicrobial activities often deployed in interspecific interactions (47). In the *Rm-Burkholderia*
231 symbiosis, an antimitotic polyketide rhizoxin is synthesized cooperatively by both partners (95,
232 108). In contrast to essential metabolites provisioned by EB to insect hosts (6, 75), rhizoxin is
233 not essential to *Rm* survival. However, it allows *Rm* to engage in pathogenesis of plants (108).
234 Such reliance of *Rm* on its EB for secondary metabolites is an important and lifestyle altering
235 evolutionary innovation, as Mucoromycota, including *Rm,* contain only a limited repertoire of
236 secondary metabolite gene clusters (55, 70, 124).
237 For vertical transmission, *Burkholderia* exploits asexual sporangiospores and sexual
238 zygospores of *Rm*, exerting different degrees of control over formation of these two types of
239 propagules (70, 96) (**Fig 3**). EB transmission via asexual sporangiospores allows for co-
240 dispersal of partner lineages. However, the extreme bottleneck size, varying from one to four
241 *Burkholderia* cells per *Rm* sporangiospore (70, 96), suggests that additional mechanisms must be
242 in place to prevent rapid genomic degeneration of EB genomes. Like other Mucoromycotina, in
243 addition to asexual proliferation via sporangiospores, the *Rm* hosts can mate and form sexual
244 zygospores (70). Consequently, it would not be unexpected for the zygospores to provide an
245 arena for mixing of symbionts associated with host parental lineages. While this hypothesis
246 remains to be tested, such mixing would be important for the retention by *Burkholderia* EB of
247 molecular evolution patterns resembling those of free-living *Burkholderia* rather than those of
248 heritable EB of insects, such as *'Candidatus* Tremblaya princeps', a closely related nutritional
249 mutualist of mealybugs (20) (**Fig 1**).
250

251 ### *2.3. Me-Mc* symbiosis

252 *Mc* is a betaproteobacterium (**Fig 1**) auxotrophic for cysteine, which is provisioned by its *Me*
253 host (86). Like other Mortierellomycotina, *Me* can be isolated from the soil and roots of trees
254 (16, 124). Importantly, not all strains of *Me* harbor *Mc* (124). The *Mc* genome of 2.6 Mb
255 represents an intermediate level of contraction compared to the genomes of its close relatives
256 *Ca*Gg and *Burkholderia* EB of *Rm* (33, 124). Elimination of *Mc* from the *Me* hyphae results in
257 improved mycelial growth (59, 124) (**Fig 3**). Changes in the colony morphology are
258 accompanied by accumulation of fatty acids that otherwise fuel *Mc* energy metabolism (124).
259 Collectively, the phenotypic effects of *Mc* elimination suggest that it is a parasite of *Me*.
260 Interestingly, the *Me-Mc* symbiosis is believed to have originated 350 MYA (124), which
261 raises questions concerning the exact nature of this association and factors that control its
262 evolutionary stability. As mentioned before, it is unlikely for strictly vertically inherited
263 parasites to persist in a host population (28, 60) unless they engage in horizontal transmission
264 (28, 60), or in a conditional mutualism (40, 61, 62, 105). As the population structure of *Mc* is
265 unknown, it is not clear whether this heritable EB undergoes horizontal transmission. However,
266 as *Me* is a heterothallic fungus in which sexual reproduction requires two compatible mates (35),
267 host mating interactions could facilitate horizontal transmission of *Mc*. It is also possible that *Mc*
268 offers some conditional services to *Me*. For example, it could protect its host against more
269 virulent horizontally transmitted parasites (61, 62). Alternatively, costs and benefits of the *Mc*
270 infection may vary spatially and temporally, and be related to the biosynthesis of secondary
271 metabolites (40, 105). Mucoromycota genomes, as we mentioned earlier, contain only a limited
272 repertoire of secondary metabolite gene clusters (55, 70, 124). In contrast, the *Mc* genome
273 harbors several of them, including one cluster encoding an insecticidal toxin, which potentially
274 could be expressed under specific environmental conditions to aid the fungal host (33, 124).
275 Such secondary metabolite complementation would resemble provision of rhizoxin by
276 *Burkholderia* EB to *Rm* (95, 108). As long as metabolic benefits provisioned by *Mc* occasionally
277 outweigh its cost to *Me*, the symbiosis could be evolutionarily stable (40, 105).
278

279 ### 2.4. AMF-*Ca*Mg symbiosis

280 Like *Ca*Gg, *Ca*Mg is a heritable EB of AMF (79). In fact, both *Ca*Gg and *Ca*Mg can coexist in
281 a single AMF host (26, 121). *Ca*Mg is an uncultivable mollicute in the *Mycoplasma pneumoniae*

282 group of the family Mycoplasmataceae (79, 80) (**Fig 2**). Even though the *Ca*Mg host range
283 extends to all major lineages of Glomeromycotina (79, 83, 121) as well as to other
284 Mucoromycota, including *Endogone* (25), not all host populations harbor this EB. The role of
285 *Ca*Mg in the biology of AMF is unknown. The *Ca*Mg genomes are highly reduced in size,
286 ranging from 0.66 to 1.23 Mb (80, 122). Consequently, *Ca*Mg is metabolically dependent on the
287 host, with the major source of energy remaining undiscovered (80, 122). Presence of the genes
288 encoding host-interactive proteins as well as genes acquired horizontally from fungi, including
289 Glomeromycotina and Mortierellomycotina (80, 122), suggests that *Ca*Mg is able to manipulate
290 its host biology.
291 While the metabolic capacity of the *Ca*Mg genomes does not offer obvious clues as to
292 whether it is a mutualist or antagonist, inferences can be made from the genome architecture (80,
293 81) and the population structure of *Ca*Mg (121). In contrast to heritable EB that act as
294 mutualists, *Ca*Mg displays uncommon genome plasticity (80, 81), remarkably high levels of
295 intrahost genetic diversity (83, 121), and population-level recombination (81, 121). These
296 patterns could be interpreted as an indication of an antagonistic arms race with the host (80, 81,
297 121). Genome plasticity in *Ca*Mg could be also viewed as a countermeasure to genomic
298 degeneration experienced by *Ca*Mg (81). *Ca*Mg, while being heritable in AMF, is derived from
299 horizontally transmitted animal-infecting mycoplasmas (80). Like its mycoplasma ancestors,
300 *Ca*Mg is missing DNA repair mechanisms, a deficiency that contributes to rapid accumulation of
301 mutations, resulting in one of fastest rates of evolution among bacteria (81). As recombination
302 and mobile genetic element (MGE) activity underlying *Ca*Mg genomic plasticity are common in
303 other mycoplasmas, *Ca*Mg must have retained these mechanisms after the host switch to fungi
304 and the transition from horizontal to vertical transmission (81). Importantly, the two
305 explanations of *Ca*Mg genomic plasticity, as an adaptation that facilitates exploitation of AMF
306 versus a countermeasure to genomic degeneration, are not mutually exclusive. Conversely, it
307 cannot be dismissed that, with genomic plasticity representing a vestige of its mycoplasma
308 ancestry, *Ca*Mg is a conventional mutualist providing yet unknown benefits to AMF. It is also
309 possible that it is a conditional mutualist that aids the host only under specific conditions (40, 61,
310 62, 105).
311 The age of the AMF-*Ca*Mg symbiosis likely pre-dates the diversification of the
312 Mucoromycota (121), attesting to considerable evolutionary stability of this heritable association.

313 Such stability could be attributed to an apparent balance between the forces contributing to
314 genomic degeneration versus plasticity experienced by *Ca*Mg (81). In particular, reconstructing
315 the patterns of accumulation of slightly deleterious mutations during *Ca*Mg evolution revealed a
316 significant acceleration of this process after ancestral *Ca*Mg had switched from horizontal to
317 vertical transmission (81). In contrast, the evolution rates along terminal phylogenetic branches
318 leading to present day *Ca*Mg (**Fig 2**) do not appear to be elevated, which suggests that, over
319 time, *Ca*Mg has refined the mechanisms responsible for purging of slightly deleterious mutations
320 (81).
321

322 ### 2.5. Why are heritable EB common in Mucoromycota?

323 Fungal-bacterial symbioses are not unique to the phylum Mucoromycota (89). However, the
324 associations formed with EB by these early divergent fungi are distinct due to a high degree of
325 co-evolution between the partners. It has been proposed that the propensity of Mucoromycota to
326 host EB is related to the aseptate nature of their hyphae, which allow free migration of EB across
327 the host mycelium (26). Another tantalizing explanation is related to the recent discovery that,
328 unlike Dikarya, early divergent fungi share with bacteria the use of 6-methyladenine (m6A)
329 DNA modification (69). 6mA is by far the most common type of DNA modifications in
330 bacteria, important for bacterial cell defense relying on restriction-modification systems (14). In
331 contrast to prokaryotes, the role of 6mA in eukaryotes has not been understood until recently (32,
332 39, 65, 66, 69, 133, 135). Recent studies revealed that 6mA is not only present in eukaryotes,
333 but plays an important role in gene expression (39, 49, 133, 135). Remarkably, the genomes of
334 early-divergent fungi contain up to 3% of 6mA, a level substantially higher than that in other
335 eukaryotes (69). Moreover, 6mA modifications appear to concentrate at the transcriptional starts
336 of expressed genes, a pattern consistent with gene activation (69). Consequently, it is attractive
337 to speculate that the shared use of 6mA DNA modification is a condition predisposing
338 Mucoromycota to bacterial manipulation, a hypothesis that remains to be tested.
339

340 ## 3. Exploring evolutionary models

341 ### 3.1. Molecular evolution rate acceleration

342 The rate of molecular evolution is expected to be higher in a population of a small effective size
343 that rapidly accumulates slightly deleterious mutations due to genetic drift compared to a

344 population of a larger size where such mutations are eliminated by natural selection (87).
345 Importantly, molecular evolution rate acceleration relative to free-living taxa is one of the
346 hallmarks of heritable EB (75), including *Ca*Gg (20). However, as we discussed earlier, with its
347 low mutation rate and a relatively large effective population size (71), *Ca*Gg appears to defy
348 predictions concerning the causes that underlie evolution rate acceleration. In fact, modeling of
349 the rates of evolution under various parameters of mutation and recombination suggested that the
350 evolution rate acceleration in *Ca*Gg is a consequence of the long-term maintenance of a largely
351 clonal population coupled with infrequent recombination (71).
352 Even though *Mc* is evolving significantly slower than *Ca*Gg, its evolution rate is
353 accelerated relative to free-living *Burkholderia* and *Burkholderia* EB of *Rm* (**Fig 1, Table 1**).
354 The genome of *Mc* contains multiple genes involved in DNA repair, including *polA, dnaQ, mutS,*
355 and *mutL* (33), which encode DNA polymerase I with proofreading activity, ε subunit of DNA
356 polymerase III with 3'→5' DNA-directed proofreading exonuclease activity, the MMRS
357 mismatch repair protein that recognizes and binds mismatched nucleotides, and MMRS
358 mismatch repair protein with endonuclease activity, respectively. While retention of these DNA
359 repair mechanisms suggests that the evolution rate acceleration in *Mc* is not caused by an
360 increased supply of mutations, the specific cause has yet to be found.
361 Unlike *Ca*Gg and *Mc, Burkholderia* EB of *Rm* evolve at a rate comparable to that of their
362 free-living relatives (20), which is somewhat surprising in a heritable EB. In the absence of
363 specific data, two hypotheses can be formulated that explain such a low evolutionary rate. First,
364 the *Rm-Burkholderia* mutualism is still at an early stage of co-evolution between the partners,
365 before the population of *Burkholderia* EB had a chance to decline in effective size and start
366 accumulating slightly deleterious mutations that disable DNA repair mechanisms. Alternatively,
367 the *Rm-Burkholderia* symbiosis is already ancient. Yet the genomes of EB are arrested at the
368 present state of evolution due to the nature of the symbiosis in which EB control host
369 reproductive biology, are free to mix, and thereby retain a large effective population size that
370 allows for symbiont-level selection. A moderate size of the *Br* genome and its retention of DNA
371 repair genes *polA, dnaQ, mutS,* and *mutL* (52) support both hypotheses. Accordingly, additional
372 work is needed to explain the low rate of molecular evolution in *Burkholderia* EB.
373 *Ca*Mg evolves at a rate that exceeds the rates observed in rapidly evolving animal-
374 associated mycoplasmas and is one of fastest among bacteria (81). As we indicated earlier, the

375 genomes of *Ca*Mg are missing genes responsible for DNA repair, which contributes to a rampant
376 accumulation of mutations (80). This mutational decay is countered by genome plasticity (80,
377 82). In turn, a dynamic equilibrium between the forces that drive the ongoing genome decay and
378 its restoration contributes to evolutionary antiquity of the AMF-*Ca*Mg symbiosis (81). The same
379 forces are also likely responsible for the ultra-rapid evolution in *Ca*Mg. Importantly, this
380 mechanism is distinct from the one governing the rapid evolution of heritable EB with
381 populations of a small effective size (73, 87). It also differs from the mechanism operating in
382 *Ca*Gg in which molecular evolution rate acceleration can be attributed to rare recombination
383 events in a predominantly clonal population with a relatively large effective size (71).
384
385 **3.2. Mutualism origins**
386 **3.2.1. Antagonism-to-mutualism transition in heritable symbioses.** In the *Rm-Burkholderia*
387 symbiosis, elimination of EB from the host mycelium abolishes asexual proliferation of the
388 fungus (96) and affects its ability to mate, either impeding sex completely or reducing the rate of
389 zygospore formation (70) (**Fig 3**). These two patterns suggest that symbionts interact with host
390 reproduction and, by doing so, they control their own transmission (70). According to one of the
391 theoretical models describing conditions required for mutualism establishment, the symbiont's
392 ability to achieve control of its own transmission is the key prerequisite for the antagonism-to-
393 mutualism transition in heritable symbioses (134). While the evolutionary history of the *Rm-*
394 *Burkholderia* mutualism is uncertain, present-day antagonistic interactions between naturally
395 EB-free (non-host) *Rm* and *Burkholderia* isolated from the host suggest that it originated as an
396 antagonism (55). The symbiont's control over own transmission is expected to facilitate
397 reciprocal selection between the partners, leading to utilization of symbiont services by the host
398 (134). In the *Rm-Burkholderia* symbiosis, these services include EB-mediated synthesis of
399 rhizoxin, which, as we discussed earlier, enables pathogenesis of plants by *Rm* (95, 108).
400 Overall, the *Rm-Burkholderia* mutualism supports the evolutionary model suggesting that a
401 heritable mutualism could evolve from an antagonism (134).
402
403 **3.2.2. Host addiction to an antagonistic symbiont.** Another theoretical model describing the
404 antagonism-to-mutualism transition, which gained support from the patterns displayed by the *Rm*
405 and *Burkholderia* partners, is the addiction model (1). According to this model, a host

406 antagonized by a parasitic symbiont will develop mechanisms that counterbalance parasite's
407 negative effects. These mechanisms may make the host addicted to the symbiont's continued
408 presence (92). In the *Rm-Burkholderia* symbiosis, the non-hosts exhibiting growth inhibition
409 when confronted by EB isolated from host fungi represent a pre-addiction stage of the fungus
410 (55). Mutualism establishment between the cured host and *Burkholderia* EB as well as bacterial
411 presence inside the host hyphae in the established symbiosis are associated with elevated
412 expression of fungal genes involved in lipid metabolism (55, 70). Activities of these enzymes
413 result in accumulation of triacylglycerol (TAG) and phosphatidylethanolamine (PE) at a ratio of
414 about 1:1 (55) (**Fig 3**). Perturbation of this ratio in favor of TAG over PE shifts the *Rm-*
415 *Burkholderia* interaction into antagonism, suggesting that the accumulation of TAG and PE at a
416 specific ratio is part of the fungal addiction syndrome to EB.
417 In addition to EB impact on host lipid metabolism, *Rm* is addicted to *Burkholderia* for
418 reproduction (70). Bacteria hijacked a component of the host's reproductive machinery by
419 gaining control over the expression of *ras2-1* (70), a gene encoding a G-protein involved in
420 asexual and sexual reproduction in other fungi (45, 46, 58). The exact mechanism of bacterial
421 control over *ras2-1* expression and the evolutionary trajectory that lead to it are unknown.
422 However, a tantalizing clue comes from observations made in yeast *Saccharomyces cerevisiae* in
423 which hyper-activation of Ras signaling induces programmed cell death (38). Accordingly, it is
424 attractive to speculate that in the ancestrally antagonistic relationship between *Rm* and
425 *Burkholderia* (55), establishing control over *ras2-1* expression by EB was an important
426 component of co-evolution between the partners, leading to adaptive changes in host regulation
427 of its Ras2-1 signaling (70).
428

429 ## 4. Fungal-bacterial symbioses: a window into the fungal biology

430 The phylum Mucoromycota is one of the least understood lineages of filamentous fungi because
431 its representatives have been remarkably recalcitrant to genetic analysis and manipulation.
432 However, recent studies of fungal-bacterial symbioses involving Mucoromycota suggest that
433 novel insights into various aspects of the Mucoromycota biology can be gleaned from a
434 systematic dissection of these associations.
435

436 ### 4.1. Lipid metabolism of Mucoromycota

437 Most Mucoromycota are oleaginous fungi that accumulate lipids to at least 20% of their biomass
438 (119). In all Mucoromycota symbioses that can be manipulated experimentally (AMF-*Ca*Gg,
439 *Me-Mc*, *Rm-Burkholderia*), symbiont elimination results in alterations of host lipid metabolism
440 (55, 59, 106, 107, 124, 126) (**Fig 3**). While the significance of these perturbations is different in
441 each of the systems, they all speak to the central role of lipid metabolism in host-EB interactions
442 involving Mucoromycota. Importantly, the examination of host responses to EB contributed to a
443 refined understanding of lipid metabolic pathways in Mucoromycota (55, 59). It also revealed
444 that some of the Mucoromycota lipid metabolic enzymes affected by EB are unique to the early
445 divergent fungi and not found in Dikarya (55).
446

447 **4.2. Reproductive biology of Mucoromycotina**

448 Reproductive dependence of *Rm* on *Burkholderia* EB established this symbiosis as a model for
449 understanding how asexual and sexual reproduction is regulated in Mucoromycotina (70, 96).
450 Several important insights have been already generated in this system. These findings include a
451 discovery that only one of the multiple paralogs of Ras2, a small GTPase central to the
452 reproductive development of other fungi, plays a role during both mating and asexual
453 proliferation of Mucoromycotina (70). In addition, a negative impact of cyclic AMP on
454 Mucoromycotina mating has been confirmed in this system (70). Lastly, candidate receptors of
455 mating pheromones unique to Mucoromycotina have been identified (70). Unlike Dikarya,
456 Mucoromycotina rely on trisporic acids and their precursors for communication between sexual
457 partners (132). While the biosynthesis of these molecules is fairly well understood (132),
458 mechanisms of their perception have been elusive.
459

460 **4.3. Reproductive biology of AMF**

461 Glomeromycotina are one of oldest and most common symbionts of plants (114). Despite their
462 close phylogenetic relationship with Mucoromycotina and Mortierellomycotina (115), they
463 display several phenotypic features that superficially set them apart from these other
464 Mucoromycota. First, unlike other predominantly saprotrophic Mucoromycota,
465 Glomeromycotina are obligate biotrophs. They have lost the fatty acid synthase, which is the
466 key enzyme complex responsible for the biosynthesis of fatty acids (118, 129). As a
467 consequence, AMF rely on their plant hosts for energy metabolism substrates. Second, although

468 cryptic recombination appears to occur in AMF (23, 24, 98), there is no direct evidence that
469 these fungi engage in a sexual process in which the union of gametangia leads to the formation
470 of zygospores typical for Mucoromycotina and Mortierellomycotina. Third, AMF do not form
471 asexual sporangiospores that are used for dispersal by most other Mucoromycota, with the
472 exception of *Endogone*. Instead, they generate large multinucleate resting spores that
473 phenotypically resemble azygospores formed by many Mucoromycotina under several specific
474 conditions (5, 37, 109, 110).
475 The apparent loss of sexual mating and sporangiospore-mediated dispersal in
476 Glomeromycotina may be attributed to selective pressures exerted by their obligate mutualism
477 with plants. In particular, genetic recombination is expected to be disfavored in mutualistic
478 microbes because new recombinant genotypes are less likely to be co-adapted to common host
479 genotypes (56, 57, 103). However, once recombination is lost, accumulation of slightly
480 deleterious mutations becomes a threat to evolutionary longevity of an asexual population (78).
481 Under such circumstances, asexual propagation becomes a key modulator of the population load
482 of deleterious mutations. Specifically, multinucleate propagules, such as those formed by AMF,
483 are more effective in purging of slightly deleterious mutations compared to uninuclear
484 propagules, like sporangiospores (43, 91, 100). Consequently, they are expected to be favored.
485 Theoretical considerations suggest that the reproductive biology of extant
486 Glomeromycotina could be solely a product of their interactions with plants. However, given the
487 role of *Burkholderia* EB in the reproductive biology of *Rm* (70, 96) and the propensity of AMF
488 for hosting diverse EB (11, 79), it is tempting to speculate that the loss of mating and
489 sporangiospore formation might have been facilitated by interactions of ancestral
490 Glomeromycotina with EB capable of modulating host reproductive biology.
491
492 **4.4. Innate immunity in Mucoromycotina**
493 The utility of the *Rm-Burkholderia* symbiosis as a model for fungal-bacterial interactions is
494 enhanced by the existence of non-host strains of *Rm* that do not harbor EB and interact
495 antagonistically with EB isolated from the host (55). Specifically, co-cultivation of cured *Rm*
496 with its own *Burkholderia* EB or *Burkholderia* isolated from other *Rm* hosts re-establishes a
497 functional symbiosis whereby bacteria populate fungal hyphae and spores (55, 70). In contrast,
498 non-host *Rm* strains do not become colonized by EB isolated from host *Rm* strains (55). A

499 similar absence of colonization was observed in other non-host Mucoromycotina such as
500 *Rhizopus oryzae* and *Mucor circinelloides* during co-cultivation with EB of *Rm* (55). Moreover,
501 the non-host fungi are antagonized by these bacteria and change their growth pattern by reducing
502 hyphal extension around bacterial colonies (55). These observations indicate that *Burkholderia*
503 isolated from *Rm* offers an excellent probe for exploring innate immunity of Mucoromycotina,
504 which, as we mentioned earlier, possess a limited repertoire of secondary metabolites that could
505 be deployed as a defense against bacterial invasions.
506

507 ## 5. Conclusions

508 Heritable symbioses formed with bacteria by the members of the phylum Mucoromycota stand
509 out among other fungal-bacterial relationships. Despite their ecological and metabolic diversity,
510 these associations are all highly co-evolved and most are ancient. They have been a source of
511 important insights into the mechanisms that stabilize heritable symbioses, control the rate of
512 molecular evolution, and enable the establishment of mutualisms. They revealed novel aspects
513 of host-microbe biology and provided a unique framework for exploring genetically intractable
514 Mucoromycota. These advances establish heritable symbioses between Mucoromycota and EB
515 as convenient and versatile research targets. Importantly, it is highly likely that many
516 Mucoromycota-EB associations with unique biological properties will soon be discovered.
517 Consequently, we expect that the current explosion of studies conducted on fungal-bacterial
518 symbioses is a good prognostic for the future expansion of this research area.
519

520 ## Summary Points

521 1. The associations of Mucoromycota with EB exemplify novel host-microbe interactions and
522 mechanisms that stabilize heritable symbioses over long evolutionary periods.
523 2. Some EB of Mucoromycota display molecular evolution rate acceleration relative to free-
524 living bacteria that cannot be attributed to accumulation of slightly deleterious mutations in a
525 population of a small effective size.
526 3. Studies of the Mucoromycota-EB symbioses allow for testing predictions of theoretical
527 models describing the origins of mutualisms.
528 4. Examination of the Mucoromycota-EB symbioses provides insights into the biology of
529 genetically intractable fungal hosts.

530 5. Novel Mucoromycota-EB symbioses are expected to be discovered.
531

532 Future Issues

533 1. What is the proximate mechanism that allows *Ca*Gg for manipulation of pre-symbiotic
534 AMF?
535 2. What is the evolutionary age of the *Rm-Burkholderia* symbiosis?
536 3. Is the *Me-Mc* symbiosis a conditional mutualism?
537 4. What is the nature of the AMF-*Ca*Mg symbiosis?
538 5. Is the shared use of m6A DNA modification predisposing Mucoromycota to harboring EB?
539

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542

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Terms and Definitions

AMF: arbuscular mycorrhizal fungi, soil fungi that colonize roots of most terrestrial plants and facilitate plant uptake of mineral nutrients from the soil in exchange for photosynthesis-derived metabolites

***Burkholderia* EB**: a heritable endosymbiotic bacterium of *Rhizopus microsporus*

***Ca*Gg**: '*Candidatus* Glomeribacter gigasporarum', a heritable endosymbiotic bacterium of arbuscular mycorrhizal fungi

***Ca*Mg**: '*Candidatus* Moeniiplasma glomeromycotorum', a heritable endosymbiotic bacterium of arbuscular mycorrhizal fungi

EB: endosymbiotic bacteria

Effective population size: a parameter that determines the rate of change in the composition of a population caused by generic drift

Genetic drift: the process of evolutionary change involving the random sampling of genes from the parental generation to produce the offspring generation

***Mc**: Mycoavidus cysteinexigens,* a heritable endosymbiotic bacterium of *Mortierella elongata*

Me: *Mortierella elongata,* a soil fungus in the subphylum Mortierellomycotina

***Rm**: Rhizopus microsporus*, a soil fungus in the subphylum Mucoromycotina

Horizontal transmission: passage of symbionts between hosts of the same generation

Mutualism: a type of symbiosis in which reciprocal exploitation provides net benefits to each partner

Symbiosis: the living together of dissimilar organisms

Vertical transmission: passage of symbionts from one host generation to the next

Zygospore: a resting spore formed by fusion of gametangia during sexual reproduction of Mucoromycota

Reference Annotations

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925 ***Burkholderia* EB interact with sexual reproduction in *Rm*. This interaction revealed**
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937 ***Ca*Gg is non-degenerative.**
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946 **This paper placed AMF into the phylum Mucoromycota and inspired our speculations**
947 **about the impact of EB on the reproductive biology of AMF.**
948
949 **Related Resources**

950 Charlesworth B. 2009. Effective population size and patterns of molecular evolution and
951 variation. *Nature Reviews Genetics* 10: 195-205
952
953

954 **Table 1.** The rate of evolution in *Mc* differs from the evolution rates in other EB and free-living
955 relatives[a].
956

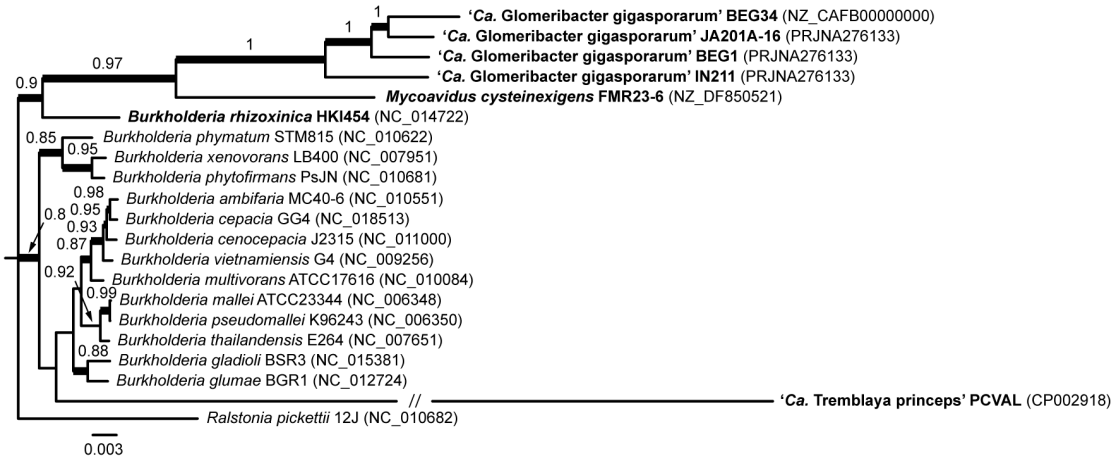
Ingroup (GenBank accession no.)	Outgroup (GenBank accession no.)	Relative rate statistic[a]
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521) '*Ca*. Glomeribacter gigasporarum' BEG34 (NZ_CAFB00000000)	*Burkholderia phytofirmans* PsJN (NC_010681)	22.88****
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521) '*Ca.* Glomeribacter gigasporarum' IN211 (PRJNA276133)	*Burkholderia phytofirmans* PsJN (NC_010681)	17.95****
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521) *Burkholderia rhizoxinica* HKI454 (NC_014722)	*Burkholderia phytofirmans* PsJN (NC_010681)	506.65****
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521) *Burkholderia phytofirmans* PsJN (NC_010681)	*Ralstonia pickettii* 12J (NC_010682)	773.73****
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521) *Burkholderia glumae* BGR1 (NC_012724)	*Ralstonia pickettii* 12J (NC_010682)	864.33****

957 [a]Results were obtained using Tajima's 1D relative rate test (116) implemented in MEGA7 (50)
958 and conducted on DNA sequences at 27 loci listed in **Fig 1**.
959 [b]The 1D relative rate statistic distribution is the same as the distribution of χ^2.
960 ****, significant at $P \leq 0.0001$.

961 ## Figure legends

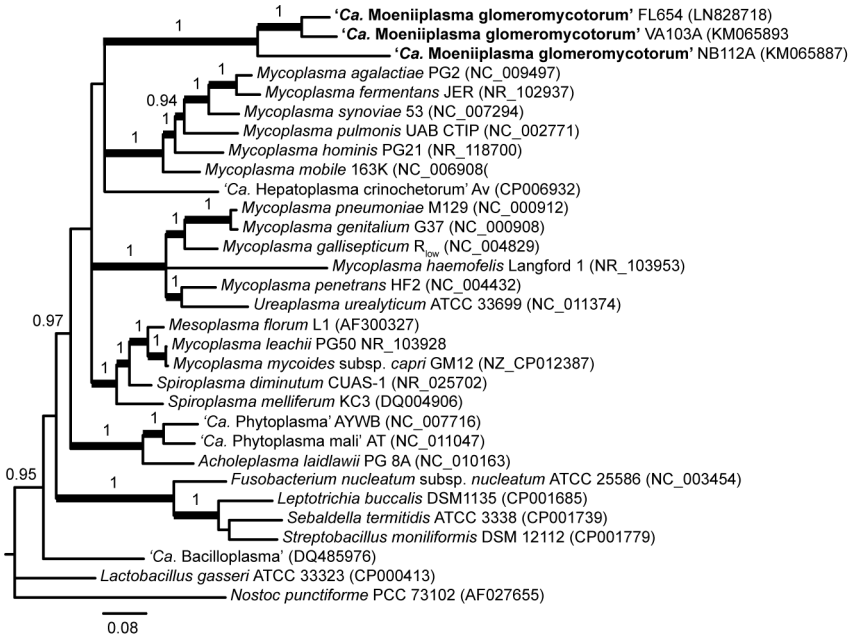
962 **Figure 1.** Evolutionary history of *CaGg, Mc* and *Burkholderia* EB reconstructed using
963 nucleotide sequences at 16S rRNA, 23S rRNA, and 25 protein-coding loci (*nusA, pyrG, rplA,*
964 *rplB, rplC, rplD, rplE, rplF, rplK, rplL, rplM, rplN, rplP, rplS, rplT, rpmA, rpoB, rpsB, rpsC,*
965 *rpsE, rpsI, rpsJ, rpsK, rpsM,* and *rpsS*). Bayesian posterior probabilities over 0.80 are shown
966 above branches. Branches with maximum likelihood bootstrap support over 70% are thickened.
967 Sequences of EB are in bold: *Ca*Gg of *Gigaspora margarita* BEG34, *Ca*Gg of *Gigaspora*
968 *margarita* JA201A-16, *Ca*Gg of *Racocetra castanea* BEG1, *Ca*Gg of *Cetraspora pellucida*
969 IN211, *Mycoavidus cysteinexigens* of *Mortierella elongata* FMR23-6, *Burkholderia rhizoxinica*
970 of *Rhizopus microsporus*, '*Ca.* Tremblaya princeps' of citrus mealybug *Planococcus citri*.
971 Figure modified from (71).
972
973 **Figure 2.** Phylogenetic placement of '*Ca.* Moeniiplasma glomeromycotorum' based on amino
974 acid sequences at 19 protein-coding loci (*dnaG, infC, nusA, rplA, rplB, rplC, rplE, rplF, rplM,*
975 *rplN, rplP, rplT, rpmA, rpsB, rpsC, rpsE, rpsJ, rpsS* and *smpB*). Bayesian posterior probabilities
976 over 0.90 are indicated above branches. Branches with maximum-likelihood bootstrap support
977 over 70 % are thickened. Sequences of *Ca*Mg are in bold: *Ca*Mg of *Dentiscutata heterogama*
978 FL654, *Ca*Mg of *Rhizophagus clarus* NB112A, *Ca*Mg of *Racocetra verrucosa* VA103A. Figure
979 modified from (79).
980
981 **Figure 3.** Cartoon representation of phenotypic effects that EB have on their Mucoromycota
982 hosts. *Ca*Gg improves germ tube extension and branching during pre-symbiotic growth of AMF
983 (left). *Burkholderia* EB interacts with *Rm* asexual sporulation and mating (center); images
984 modified from (70). *Mc* reduces colony expansion in *Me* (right). Red ovals represent EB; fungal
985 structures, including AMF spores and germ tubes, *Rm* zygospores and sporangia with
986 sporangiospores, and *Me* mycelia, are not drawn to scale. FA, fatty acids.

987 **Figure 1**



988

989 **Figure 2**



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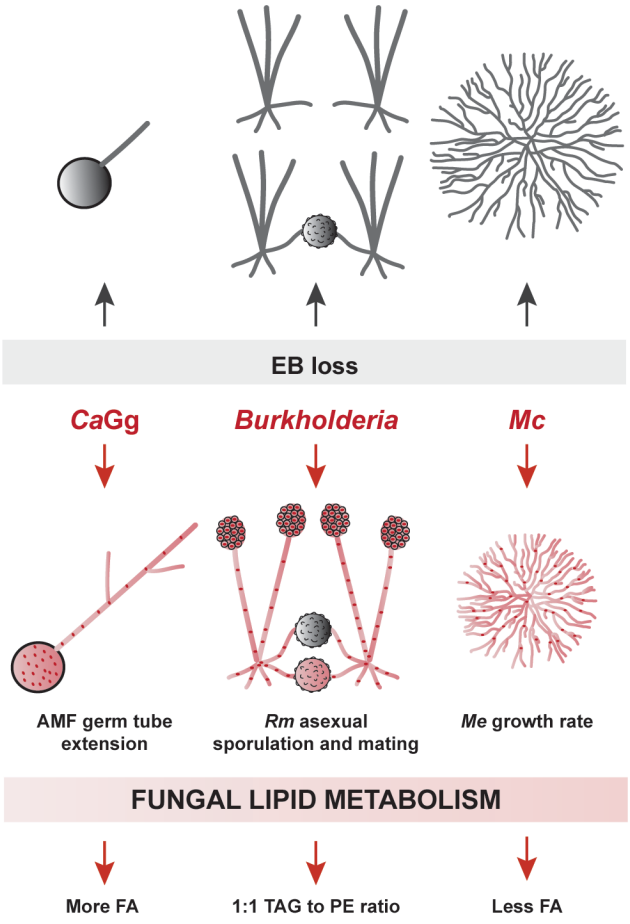
991 **Figure 3**



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