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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

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

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

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

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

symbionts and the symbiosis as a whole. Symbiont population subdivisions, transmission bottlenecks, and clonality reduce the effective size of a symbiont population and magnify the impact of genetic drift relative to natural selection (90). As a consequence, symbiont populations become vulnerable to accumulation of slightly deleterious (88) and eventual extinction (78). In heritable EB, this process is associated with genomic decay and reduction of the genome size (7, 67, 74, 84). Such degenerative genome evolution has been observed empirically in free-living bacteria evolving under conditions of a small effective population size (84), and inferred from molecular evolution patterns in multiple heritable EB that provision insects with essential metabolites (7, 67, 74). Another important consequence of degenerative evolution in heritable EB is acceleration of the molecular evolution rate compared to free-living relatives (73, 87).

Remarkably, most of the Mucoromycota-EB symbioses are ancient (15, 72, 121, 124). Two are mutualisms (AMF-*Ca*Gg and *Rm-Burkholderia*), one is an antagonism (*Me-Mc*), and one remains unresolved in terms of partner fitness outcomes (AMF-*Ca*Mg). As a consequence, Mucoromycota-EB associations exemplify diverse mechanisms that control evolutionary stability and longevity in symbioses with vertically transmitted EB. Moreover, with the exception of *Burkholderia* EB, symbionts of Mucoromycota appear to evolve faster than their free-living relatives (20, 81), and thus offer insights into how molecular rate acceleration is achieved in EB with different lifestyles. In addition, these symbioses allow for exploring theoretical predictions that specify conditions necessary for mutualisms to arise. Many such predictions have not been tested rigorously because very few heritable partnerships outside Mucoromycota are amenable to experimental manipulation.

In this review, we summarize key features of Mucoromycota-EB partnerships, use molecular evolution patterns apparent in these symbioses to speculate about uncertainties surrounding some of their aspects, describe how studies of the Mucoromycota-EB associations inform and validate theoretical models of symbiosis evolution, and detail how they can be used to generate specific insights into the facets of host biology that historically have been recalcitrant to investigation. In the process, we highlight future research directions.

2. Host-symbiont biology and symbiosis stability

2.1. AMF-*Ca*Gg mutualism

*Ca*Gg is a betaproteobacterium (**Fig 1**) and a mutualist of AMF from the family Gigasporaceae (9, 64, 72). AMF are obligate biotrophs that colonize roots of most terrestrial plants and facilitate plant uptake of mineral nutrients from the soil (114) in exchange for photosynthesis-derived monosaccharides (41) and fatty acids (17, 44, 48, 63) coming from the plant. The association that AMF form with plants, arbuscular mycorrhiza, dates back to the Early Devonian, 400 MYA (97), and is one of the oldest mutualisms on the planet. AMF are increasingly recognized in agronomy as sustainable biofertilizers of the future (127).

*Ca*Gg is vertically transmitted through AMF generations (10) and shows variable distribution across host populations, with some AMF individuals harboring the EB and some being *Ca*Gg-free (12, 72). This pattern suggests that *Ca*Gg is a nonessential partner of AMF. Serial sub-culturing of AMF can lead to elimination of *Ca*Gg under laboratory conditions (64). For AMF, phenotypic consequences of *Ca*Gg loss include reduced elongation and branching of pre-symbiotic hyphae that emerge from spores in the presence of plant roots (64) (**Fig 3**). At the subcellular level, the absence of *Ca*Gg from pre-symbiotic hosts is accompanied by a decline in the volume of lipid droplets present in fungal cells (64). Without *Ca*Gg, spore fatty acids become less abundant, with particular depletion of palmitic acid (106). Pre-symbiotic AMF are unable to synthesize palmitate (123) because they lack genes encoding the fatty acid synthase enzyme complex (118, 129). Consequently, the efficiency of how spore energy reserves are utilized is important for the AMF ability to associate with a plant host. In fungi cured of *Ca*Gg, reductions in lipid droplet volume and fatty acid abundance are accompanied by elevated expression of genes and proteins involved in beta-oxidation of fatty acids and the pentose phosphate pathway, suggesting a shift towards pathways that provide reducing power (126). In contrast, pre-symbiotic fungi harboring *Ca*Gg acquire their reducing power due to elevated mitochondrial oxidative phosphorylation and ATP biosynthesis (107, 126). These increases are associated with respiration rates 50% higher than in the cured fungi (126). Overall, *Ca*Gg appears to interact with AMF energy metabolism in ways that mobilize ATP and fuel pre-symbiotic growth. Interestingly, similar effects are caused by strigolactones, plant hormones that AMF perceive and respond to by enhancing hyphal branching, proliferation of mitochondria and increasing respiration (8, 54). Remarkably, the strigolactone treatment also induces a 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.

*Ca*Gg is transmitted uniparentally, along clonal lineages of its AMF hosts. AMF show no direct evidence of sexual mating and rely on large multinucleate spores for asexual proliferation. Intrahost populations of *Ca*Gg are genetically uniform (72). Such genetic homogeneity could be attributed to a rate of mutation accumulation in *Ca*Gg that is comparable to that of free-living bacteria (71). This low mutation rate (71) and a relatively large effective population size in *Ca*Gg (71) are also likely to be responsible for the extraordinary evolutionary longevity of the AMF-*Ca*Gg symbiosis, which dates back to the Early Devonian (72).

What remains uncertain are the forces that allow *Ca*Gg to maintain a relatively large population size. It is possible that the ultimate cause is related to the nature of *Ca*Gg association with AMF. *Ca*Gg services are not essential to AMF, or, in other words, AMF are only facultatively reliant on *Ca*Gg (64, 72). Such reliance suggests that fitness benefits of carrying EB vary depending on specific conditions, with certain environments favoring EB presence and others selecting against it (101, 102). A variable selective landscape is expected to support retention of genetic competence for horizontal transmission and recombination (85), which are present in *Ca*Gg (71, 72). However, the specific environmental factors responsible for AMF facultative rather than obligate dependence on *Ca*Gg are unknown. It could be speculated that these factors are related to conditions affecting pre-symbiotic activities of obligately biotrophic AMF, such as the number of spore germination attempts and the extent of hyphal proliferation.

2.2. *Rm-Burkholderia* mutualism

Rm, like most other Mucoromycotina, is a saprotroph that also can act as an opportunistic pathogen of plants and humans (93, 108). While multiple *Burkholderia* EB species have been found in different isolates of this fungus, such as *Burkholderia rhizoxinica, Br* (51, 68, 95, 96, 113, 125), *Burkholderia endofungorum* (94) and *Burkholderia* sp. (55, 70) (**Fig 1**), no *Burkholderia* EB have been found in other Mucoromycotina (111). Moreover, even within *Rm* some strains do not harbor these EB (55, 93).

The *Rm-Burkholderia* mutualism has become a model for understanding fungal-bacterial symbioses because it can be manipulated experimentally, hosts can be cured of symbionts, and partners separated and reassembled back into a functional symbiosis (51, 55, 68, 70, 95). This versatility is related to the genomic makeup of *Burkholderia* EB. The 3.75 Mb genome of *Br* (52) supports functional capabilities important for *Burkholderia* EB persistence outside the host

cellular environment and host recolonization as well as endosymbiotic lifestyle and vertical transmission (51, 68, 95). Recolonization of the fungal mycelium is possible due to the activity of *Burkholderia* secretion systems. These systems include the Type II Secretion System, which translocates fungal cell wall-degrading enzymes chitinase and chitosinase (68) as well as the Type III Secretion System (51), which delivers effectors for host manipulation directly into the host cytoplasm (22). The establishment of symbiosis is associated with alterations of the *Rm* lipid metabolism (55). Host lipids are also important for the maintenance of the symbiosis, as they likely provide substrates for *Burkholderia* energy metabolism (52, 53) (**Fig 3**).

Nearly 10% of the *Br* genome is comprised of secondary metabolite gene clusters (52, 53). Secondary metabolites are low molecular weight compounds with potent physiological and antimicrobial activities often deployed in interspecific interactions (47). In the *Rm-Burkholderia* symbiosis, an antimitotic polyketide rhizoxin is synthesized cooperatively by both partners (95, 108). In contrast to essential metabolites provisioned by EB to insect hosts (6, 75), rhizoxin is not essential to *Rm* survival. However, it allows *Rm* to engage in pathogenesis of plants (108). Such reliance of *Rm* on its EB for secondary metabolites is an important and lifestyle altering evolutionary innovation, as Mucoromycota, including *Rm,* contain only a limited repertoire of secondary metabolite gene clusters (55, 70, 124).

For vertical transmission, *Burkholderia* exploits asexual sporangiospores and sexual zygospores of *Rm*, exerting different degrees of control over formation of these two types of propagules (70, 96) (**Fig 3**). EB transmission via asexual sporangiospores allows for co-dispersal of partner lineages. However, the extreme bottleneck size, varying from one to four *Burkholderia* cells per *Rm* sporangiospore (70, 96), suggests that additional mechanisms must be in place to prevent rapid genomic degeneration of EB genomes. Like other Mucoromycotina, in addition to asexual proliferation via sporangiospores, the *Rm* hosts can mate and form sexual zygospores (70). Consequently, it would not be unexpected for the zygospores to provide an arena for mixing of symbionts associated with host parental lineages. While this hypothesis remains to be tested, such mixing would be important for the retention by *Burkholderia* EB of molecular evolution patterns resembling those of free-living *Burkholderia* rather than those of heritable EB of insects, such as *'Candidatus* Tremblaya princeps', a closely related nutritional mutualist of mealybugs (20) (**Fig 1**).

2.3. Me-Mc symbiosis

Mc is a betaproteobacterium (**Fig 1**) auxotrophic for cysteine, which is provisioned by its *Me* host (86). Like other Mortierellomycotina, *Me* can be isolated from the soil and roots of trees (16, 124). Importantly, not all strains of *Me* harbor *Mc* (124). The *Mc* genome of 2.6 Mb represents an intermediate level of contraction compared to the genomes of its close relatives *Ca*Gg and *Burkholderia* EB of *Rm* (33, 124). Elimination of *Mc* from the *Me* hyphae results in improved mycelial growth (59, 124) (**Fig 3**). Changes in the colony morphology are accompanied by accumulation of fatty acids that otherwise fuel *Mc* energy metabolism (124). Collectively, the phenotypic effects of *Mc* elimination suggest that it is a parasite of *Me*.

Interestingly, the *Me-Mc* symbiosis is believed to have originated 350 MYA (124), which raises questions concerning the exact nature of this association and factors that control its evolutionary stability. As mentioned before, it is unlikely for strictly vertically inherited parasites to persist in a host population (28, 60) unless they engage in horizontal transmission (28, 60), or in a conditional mutualism (40, 61, 62, 105). As the population structure of *Mc* is unknown, it is not clear whether this heritable EB undergoes horizontal transmission. However, as *Me* is a heterothallic fungus in which sexual reproduction requires two compatible mates (35), host mating interactions could facilitate horizontal transmission of *Mc*. It is also possible that *Mc* offers some conditional services to *Me*. For example, it could protect its host against more virulent horizontally transmitted parasites (61, 62). Alternatively, costs and benefits of the *Mc* infection may vary spatially and temporally, and be related to the biosynthesis of secondary metabolites (40, 105). Mucoromycota genomes, as we mentioned earlier, contain only a limited repertoire of secondary metabolite gene clusters (55, 70, 124). In contrast, the *Mc* genome harbors several of them, including one cluster encoding an insecticidal toxin, which potentially could be expressed under specific environmental conditions to aid the fungal host (33, 124). Such secondary metabolite complementation would resemble provision of rhizoxin by *Burkholderia* EB to *Rm* (95, 108). As long as metabolic benefits provisioned by *Mc* occasionally outweigh its cost to *Me*, the symbiosis could be evolutionarily stable (40, 105).

2.4. AMF-*Ca*Mg symbiosis

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

group of the family Mycoplasmataceae (79, 80) (**Fig 2**). Even though the *Ca*Mg host range extends to all major lineages of Glomeromycotina (79, 83, 121) as well as to other Mucoromycota, including *Endogone* (25), not all host populations harbor this EB. The role of *Ca*Mg in the biology of AMF is unknown. The *Ca*Mg genomes are highly reduced in size, ranging from 0.66 to 1.23 Mb (80, 122). Consequently, *Ca*Mg is metabolically dependent on the host, with the major source of energy remaining undiscovered (80, 122). Presence of the genes encoding host-interactive proteins as well as genes acquired horizontally from fungi, including Glomeromycotina and Mortierellomycotina (80, 122), suggests that *Ca*Mg is able to manipulate its host biology.

While the metabolic capacity of the *Ca*Mg genomes does not offer obvious clues as to whether it is a mutualist or antagonist, inferences can be made from the genome architecture (80, 81) and the population structure of *Ca*Mg (121). In contrast to heritable EB that act as mutualists, *Ca*Mg displays uncommon genome plasticity (80, 81), remarkably high levels of intrahost genetic diversity (83, 121), and population-level recombination (81, 121). These patterns could be interpreted as an indication of an antagonistic arms race with the host (80, 81, 121). Genome plasticity in *Ca*Mg could be also viewed as a countermeasure to genomic degeneration experienced by *Ca*Mg (81). *Ca*Mg, while being heritable in AMF, is derived from horizontally transmitted animal-infecting mycoplasmas (80). Like its mycoplasma ancestors, *Ca*Mg is missing DNA repair mechanisms, a deficiency that contributes to rapid accumulation of mutations, resulting in one of fastest rates of evolution among bacteria (81). As recombination and mobile genetic element (MGE) activity underlying *Ca*Mg genomic plasticity are common in other mycoplasmas, *Ca*Mg must have retained these mechanisms after the host switch to fungi and the transition from horizontal to vertical transmission (81). Importantly, the two explanations of *Ca*Mg genomic plasticity, as an adaptation that facilitates exploitation of AMF versus a countermeasure to genomic degeneration, are not mutually exclusive. Conversely, it cannot be dismissed that, with genomic plasticity representing a vestige of its mycoplasma ancestry, *Ca*Mg is a conventional mutualist providing yet unknown benefits to AMF. It is also possible that it is a conditional mutualist that aids the host only under specific conditions (40, 61, 62, 105).

The age of the AMF-*Ca*Mg symbiosis likely pre-dates the diversification of the Mucoromycota (121), attesting to considerable evolutionary stability of this heritable association.

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

2.5. Why are heritable EB common in Mucoromycota?

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

3. Exploring evolutionary models

3.1. Molecular evolution rate acceleration

The rate of molecular evolution is expected to be higher in a population of a small effective size 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

genomes of *Ca*Mg are missing genes responsible for DNA repair, which contributes to a rampant accumulation of mutations (80). This mutational decay is countered by genome plasticity (80, 82). In turn, a dynamic equilibrium between the forces that drive the ongoing genome decay and its restoration contributes to evolutionary antiquity of the AMF-*Ca*Mg symbiosis (81). The same forces are also likely responsible for the ultra-rapid evolution in *Ca*Mg. Importantly, this mechanism is distinct from the one governing the rapid evolution of heritable EB with populations of a small effective size (73, 87). It also differs from the mechanism operating in *Ca*Gg in which molecular evolution rate acceleration can be attributed to rare recombination events in a predominantly clonal population with a relatively large effective size (71).

3.2. Mutualism origins

3.2.1. Antagonism-to-mutualism transition in heritable symbioses. In the *Rm-Burkholderia* symbiosis, elimination of EB from the host mycelium abolishes asexual proliferation of the fungus (96) and affects its ability to mate, either impeding sex completely or reducing the rate of zygospore formation (70) (**Fig 3**). These two patterns suggest that symbionts interact with host reproduction and, by doing so, they control their own transmission (70). According to one of the theoretical models describing conditions required for mutualism establishment, the symbiont's ability to achieve control of its own transmission is the key prerequisite for the antagonism-to-mutualism transition in heritable symbioses (134). While the evolutionary history of the *Rm-Burkholderia* mutualism is uncertain, present-day antagonistic interactions between naturally EB-free (non-host) *Rm* and *Burkholderia* isolated from the host suggest that it originated as an antagonism (55). The symbiont's control over own transmission is expected to facilitate reciprocal selection between the partners, leading to utilization of symbiont services by the host (134). In the *Rm-Burkholderia* symbiosis, these services include EB-mediated synthesis of rhizoxin, which, as we discussed earlier, enables pathogenesis of plants by *Rm* (95, 108). Overall, the *Rm-Burkholderia* mutualism supports the evolutionary model suggesting that a heritable mutualism could evolve from an antagonism (134).

3.2.2. Host addiction to an antagonistic symbiont. Another theoretical model describing the antagonism-to-mutualism transition, which gained support from the patterns displayed by the *Rm* and *Burkholderia* partners, is the addiction model (1). According to this model, a host

antagonized by a parasitic symbiont will develop mechanisms that counterbalance parasite's negative effects. These mechanisms may make the host addicted to the symbiont's continued presence (92). In the *Rm-Burkholderia* symbiosis, the non-hosts exhibiting growth inhibition when confronted by EB isolated from host fungi represent a pre-addiction stage of the fungus (55). Mutualism establishment between the cured host and *Burkholderia* EB as well as bacterial presence inside the host hyphae in the established symbiosis are associated with elevated expression of fungal genes involved in lipid metabolism (55, 70). Activities of these enzymes result in accumulation of triacylglycerol (TAG) and phosphatidylethanolamine (PE) at a ratio of about 1:1 (55) (**Fig 3**). Perturbation of this ratio in favor of TAG over PE shifts the *Rm-Burkholderia* interaction into antagonism, suggesting that the accumulation of TAG and PE at a specific ratio is part of the fungal addiction syndrome to EB.

In addition to EB impact on host lipid metabolism, *Rm* is addicted to *Burkholderia* for reproduction (70). Bacteria hijacked a component of the host's reproductive machinery by gaining control over the expression of *ras2-1* (70), a gene encoding a G-protein involved in asexual and sexual reproduction in other fungi (45, 46, 58). The exact mechanism of bacterial control over *ras2-1* expression and the evolutionary trajectory that lead to it are unknown. However, a tantalizing clue comes from observations made in yeast *Saccharomyces cerevisiae* in which hyper-activation of Ras signaling induces programmed cell death (38). Accordingly, it is attractive to speculate that in the ancestrally antagonistic relationship between *Rm* and *Burkholderia* (55), establishing control over *ras2-1* expression by EB was an important component of co-evolution between the partners, leading to adaptive changes in host regulation of its Ras2-1 signaling (70).

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

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

4.1. Lipid metabolism of Mucoromycota

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

4.2. Reproductive biology of Mucoromycotina

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

4.3. Reproductive biology of AMF

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

cryptic recombination appears to occur in AMF (23, 24, 98), there is no direct evidence that these fungi engage in a sexual process in which the union of gametangia leads to the formation of zygospores typical for Mucoromycotina and Mortierellomycotina. Third, AMF do not form asexual sporangiospores that are used for dispersal by most other Mucoromycota, with the exception of *Endogone*. Instead, they generate large multinucleate resting spores that phenotypically resemble azygospores formed by many Mucoromycotina under several specific conditions (5, 37, 109, 110).

The apparent loss of sexual mating and sporangiospore-mediated dispersal in Glomeromycotina may be attributed to selective pressures exerted by their obligate mutualism with plants. In particular, genetic recombination is expected to be disfavored in mutualistic microbes because new recombinant genotypes are less likely to be co-adapted to common host genotypes (56, 57, 103). However, once recombination is lost, accumulation of slightly deleterious mutations becomes a threat to evolutionary longevity of an asexual population (78). Under such circumstances, asexual propagation becomes a key modulator of the population load of deleterious mutations. Specifically, multinucleate propagules, such as those formed by AMF, are more effective in purging of slightly deleterious mutations compared to uninuclear propagules, like sporangiospores (43, 91, 100). Consequently, they are expected to be favored.

Theoretical considerations suggest that the reproductive biology of extant Glomeromycotina could be solely a product of their interactions with plants. However, given the role of *Burkholderia* EB in the reproductive biology of *Rm* (70, 96) and the propensity of AMF for hosting diverse EB (11, 79), it is tempting to speculate that the loss of mating and sporangiospore formation might have been facilitated by interactions of ancestral Glomeromycotina with EB capable of modulating host reproductive biology.

4.4. Innate immunity in Mucoromycotina

The utility of the *Rm-Burkholderia* symbiosis as a model for fungal-bacterial interactions is enhanced by the existence of non-host strains of *Rm* that do not harbor EB and interact antagonistically with EB isolated from the host (55). Specifically, co-cultivation of cured *Rm* with its own *Burkholderia* EB or *Burkholderia* isolated from other *Rm* hosts re-establishes a functional symbiosis whereby bacteria populate fungal hyphae and spores (55, 70). In contrast, non-host *Rm* strains do not become colonized by EB isolated from host *Rm* strains (55). A

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

5. Conclusions

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

Summary Points

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

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

Future Issues

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

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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

55. Lastovetsky OA, Gaspar ML, Mondo SJ, LaButti KM, Sandor L, et al. 2016. Lipid metabolic changes in an early divergent fungus govern the establishment of a mutualistic symbiosis with endobacteria. *Proceedings of the National Academy of Sciences of the United States of America* 113: 15102-07

Host lipid metabolism plays a role in the establishment of the *Rm-Burkholderia* mutualism. Some lipid metabolic genes active in this process are only found in early divergent fungi.

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Burkholderia **EB interact with sexual reproduction in *Rm*. This interaction revealed candidate receptors of trisporic acids, mating pheromones unique to Mucoromycotina.**

69. Mondo SJ, Dannebaum RO, Kuo RC, Louie KB, Bewick AJ, et al. 2017. Widespread adenine N6-methylation of active genes in fungi. *Nature Genetics* 49: 964-68

The m6A DNA modification, which is common in bacteria, is also found in early divergent fungi and plays a role in gene activation.

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In contrast to degenerately evolving heritable essential EB of insects, genome evolution in *Ca*Gg is non-degenerative.

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Genome plasticity counters genomic degeneration in *Ca*Mg.

115. Spatafora JW, Chang Y, Benny GL, Lazarus K, Smith ME, et al. 2016. A phylum-level phylogenetic classification of zygomycete fungi based on genome-scale data. *Mycologia* 108: 1028-46

This paper placed AMF into the phylum Mucoromycota and inspired our speculations about the impact of EB on the reproductive biology of AMF.

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 $P \leq 0.0001$.

Figure legends

Figure 1. Evolutionary history of *CaGg, Mc* and *Burkholderia* EB reconstructed using nucleotide sequences at 16S rRNA, 23S rRNA, and 25 protein-coding loci (*nusA, pyrG, rplA, rplB, rplC, rplD, rplE, rplF, rplK, rplL, rplM, rplN, rplP, rplS, rplT, rpmA, rpoB, rpsB, rpsC, rpsE, rpsI, rpsJ, rpsK, rpsM,* and *rpsS*). Bayesian posterior probabilities over 0.80 are shown above branches. Branches with maximum likelihood bootstrap support over 70% are thickened. Sequences of EB are in bold: *Ca*Gg of *Gigaspora margarita* BEG34, *Ca*Gg of *Gigaspora margarita* JA201A-16, *Ca*Gg of *Racocetra castanea* BEG1, *Ca*Gg of *Cetraspora pellucida* IN211, *Mycoavidus cysteinexigens* of *Mortierella elongata* FMR23-6, *Burkholderia rhizoxinica* of *Rhizopus microsporus*, '*Ca.* Tremblaya princeps' of citrus mealybug *Planococcus citri*. Figure modified from (71).

Figure 2. Phylogenetic placement of '*Ca.* Moeniiplasma glomeromycotorum' based on amino acid sequences at 19 protein-coding loci (*dnaG, infC, nusA, rplA, rplB, rplC, rplE, rplF, rplM, rplN, rplP, rplT, rpmA, rpsB, rpsC, rpsE, rpsJ, rpsS* and *smpB*). Bayesian posterior probabilities over 0.90 are indicated above branches. Branches with maximum-likelihood bootstrap support over 70 % are thickened. Sequences of *Ca*Mg are in bold: *Ca*Mg of *Dentiscutata heterogama* FL654, *Ca*Mg of *Rhizophagus clarus* NB112A, *Ca*Mg of *Racocetra verrucosa* VA103A. Figure modified from (79).

Figure 3. Cartoon representation of phenotypic effects that EB have on their Mucoromycota hosts. *Ca*Gg improves germ tube extension and branching during pre-symbiotic growth of AMF (left). *Burkholderia* EB interacts with *Rm* asexual sporulation and mating (center); images modified from (70). *Mc* reduces colony expansion in *Me* (right). Red ovals represent EB; fungal structures, including AMF spores and germ tubes, *Rm* zygospores and sporangia with sporangiospores, and *Me* mycelia, are not drawn to scale. FA, fatty acids.

Figure 1

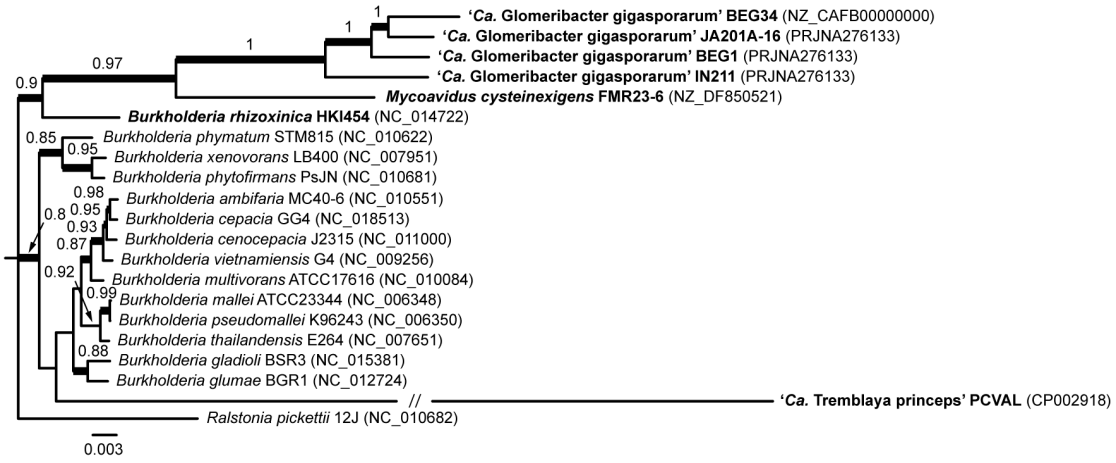
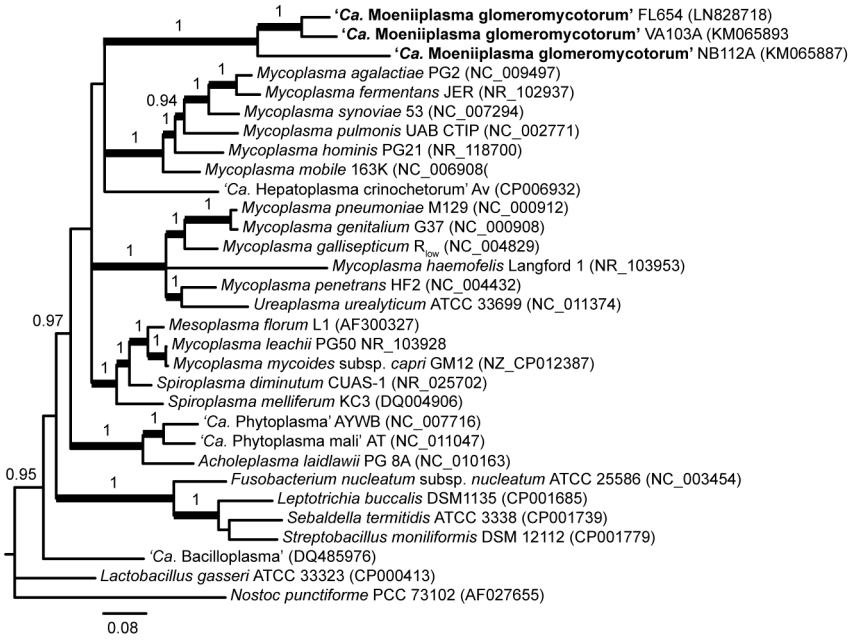
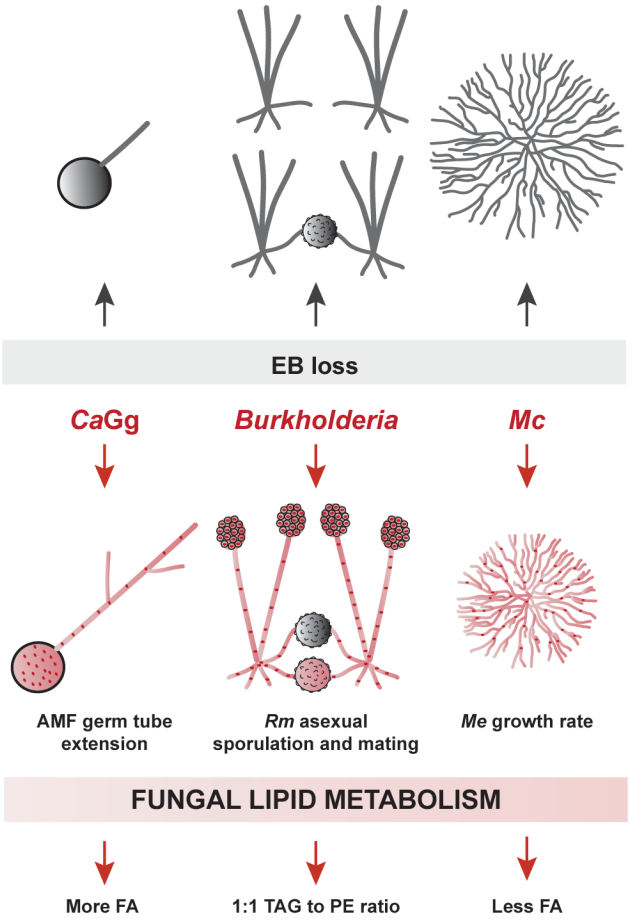

'Ca. Glomeribacter gigasporarum' BEG34 (NZ_CAFB00000000)
'Ca. Glomeribacter gigasporarum' JA201A-16 (PRJNA276133)
'Ca. Glomeribacter gigasporarum' BEG1 (PRJNA276133)
'Ca. Glomeribacter gigasporarum' IN211 (PRJNA276133)
Mycoavidus cysteinexigens FMR23-6 (NZ_DF850521)
Burkholderia rhizoxinica HKI454 (NC_014722)
Burkholderia phymatum STM815 (NC_010622)
Burkholderia xenovorans LB400 (NC_007951)
Burkholderia phytofirmans PsJN (NC_010681)
Burkholderia ambifaria MC40-6 (NC_010551)
Burkholderia cepacia GG4 (NC_018513)
Burkholderia cenocepacia J2315 (NC_011000)
Burkholderia vietnamiensis G4 (NC_009256)
Burkholderia multivorans ATCC17616 (NC_010084)
Burkholderia mallei ATCC23344 (NC_006348)
Burkholderia pseudomallei K96243 (NC_006350)
Burkholderia thailandensis E264 (NC_007651)
Burkholderia gladioli BSR3 (NC_015381)
Burkholderia glumae BGR1 (NC_012724)
'Ca. Tremblaya princeps' PCVAL (CP002918)
Ralstonia pickettii 12J (NC_010682)
0.003

Figure 2


'Ca. Moeniiplasma glomeromycotorum' FL654 (LN828718)
'Ca. Moeniiplasma glomeromycotorum' VA103A (KM065893
'Ca. Moeniiplasma glomeromycotorum' NB112A (KM065887)
Mycoplasma agalactiae PG2 (NC_009497)
Mycoplasma fermentans JER (NR_102937)
Mycoplasma synoviae 53 (NC_007294)
Mycoplasma pulmonis UAB CTIP (NC_002771)
Mycoplasma hominis PG21 (NR_118700)
Mycoplasma mobile 163K (NC_006908(
'Ca. Hepatoplasma crinochetorum' Av (CP006932)
Mycoplasma pneumoniae M129 (NC_000912)
Mycoplasma genitalium G37 (NC_000908)
Mycoplasma gallisepticum R$_{low}$ (NC_004829)
Mycoplasma haemofelis Langford 1 (NR_103953)
Mycoplasma penetrans HF2 (NC_004432)
Ureaplasma urealyticum ATCC 33699 (NC_011374)
Mesoplasma florum L1 (AF300327)
Mycoplasma leachii PG50 NR_103928
Mycoplasma mycoides subsp. capri GM12 (NZ_CP012387)
Spiroplasma diminutum CUAS-1 (NR_025702)
Spiroplasma melliferum KC3 (DQ004906)
'Ca. Phytoplasma' AYWB (NC_007716)
'Ca. Phytoplasma mali' AT (NC_011047)
Acholeplasma laidlawii PG 8A (NC_010163)
Fusobacterium nucleatum subsp. nucleatum ATCC 25586 (NC_003454)
Leptotrichia buccalis DSM1135 (CP001685)
Sebaldella termitidis ATCC 3338 (CP001739)
Streptobacillus moniliformis DSM 12112 (CP001779)
'Ca. Bacilloplasma' (DQ485976)
Lactobacillus gasseri ATCC 33323 (CP000413)
Nostoc punctiforme PCC 73102 (AF027655)
0.08

991 **Figure 3**



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