A complex symbiosis involving within species variation in the response of *Dictyostelium* amoebae to *Burkholderia* bacteria

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Running Title: Conflict in the *Dictyostelium-Burkholderia* symbiosis

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Abstract

Recent symbioses, particularly facultative ones, are well suited for unravelling the evolutionary give and take between partners. Here we look at variation in wild-collected samples of the social amoeba *Dictyostelium discoideum* and their relationships with bacterial symbionts, *Burkholderia hayleyella* and *Burkholderia agricolaris*. Only about a third of field-collected amoebae carry a symbiont. We cured and cross-infected *D. discoideum* hosts with different symbiont association histories and then compared the responses of the amoebae to each symbiont type. Before curing, field-collected clones did not vary significantly in overall fitness, but infected hosts produced morphologically different multicellular structures. After curing and re-infecting, host fitness declined overall. However, natural *B. hayleyella* hosts suffered fewer fitness costs when re-infected with *B. hayleyella,* indicating that they have evolved mechanisms to tolerate their naturally acquired symbiont. Exploring relationships between endosymbionts and hosts that vary within species may also reveal much about disease dynamics.

INTRODUCTION

Relationships are complicated because each party has evolved to maximize its own interests. Mutualisms arise when different parties have abilities or resources easy for them and hard for the partner, under conditions where exploitation is controlled (2015). Mutualisms where one party is microbial fall under the general category of symbiosis. Symbioses are rife with potential conflict (Dale and Moran, 2006; Estrela et al., 2016; Garcia and Gerardo, 2014; Moran, 2007; Oliver et al., 2005). Despite this, symbiotic relationships are pervasive and persistent (Douglas, 2008; Moran et al., 1993; Wernegreen, 2017; Werner et al., 2015). The stability and ubiquity of these interactions implies that conflict can be managed or minimized by partners over multiple generations. However, stability does not imply stagnation. Often, we only observe a close-up of these relationships from brief snap-shots. If we zoom out over evolutionary time, we might better illuminate an ongoing tug-of-war (Ferdy and Godelle, 2005; Hosokawa et al., 2016). Indeed, the view that symbiotic associations fluctuate along a mutualism-to-parasitism continuum is increasingly appreciated (McFall-Ngai et al., 2013).

Intracellular endosymbiosis involves a particularly intricate dance between partners. Intracellular endosymbionts must invade, survive, and replicate within host cells and move to new hosts. To exploit their host niche, mutualistic endosymbionts evolve specialized lifestyles that parallel those of intracellular pathogens (Casadevall, 2008; McCutcheon and Moran, 2011; Soto et al., 2009). Despite initial exploitative strategies, some endosymbionts become beneficial or even obligate for host survival, a situation common for many insect nutritional endosymbioses (Douglas, 2009). Strict vertical transmission of endosymbionts typically promotes a more tranquil relationship as the

evolutionary fates of the two parties become increasingly intertwined (Ferdy and Godelle, 2005; Hosokawa et al., 2016). Host dependency also often leads to the reduction in size of symbiont genomes as they become more streamlined for life within their host, something true for both beneficial and pathogenic endosymbionts (Dale and Moran, 2006). Some symbionts are not vertically transmitted but instead are acquired horizontally, a category that includes facultative symbioses. These are more likely to retain active conflicts. This has been experimentally demonstrated by manipulating the jellyfish symbiont *Symbiodinium microadriaticum* towards solely vertical or horizontal transmission modes, leading to the evolution of more mutualistic or parasitic lineages respectively (Sachs and Wilcox, 2006). Though horizontal transmission can favor symbionts that behave more parasitically, selection can favor hosts that employ more severe countermeasures to limit symbiont entry or growth (Nyholm and McFall-Ngai, 2004; Ratzka et al., 2012; Reynolds and Rolff, 2008). Indeed, host-driven control can be extreme, as demonstrated by the ability of *Paramecium bursaria* to manipulate its facultative nutritional symbiont (*Chlorella* sp.) in a relationship that provides no apparent benefit for the imprisoned symbiont (Lowe et al., 2016).

Symbiotic relationships may be positive or negative under different environmental conditions (Leung and Poulin, 2008; Pérez-Brocal et al., 2011). An interesting example of context dependency occurs in the *Acyrthosiphon pisum-Hamiltonella defensa* symbiosis (Oliver et al., 2003, 2005). *H. defensa* infection confers host resistance to parasitoid attack, with resistance being greater for hosts co-infected with *Serratia symbiotica* (Oliver et al., 2006). However, co-infection comes at a high fecundity cost, such that in parasitoid free environments, host fitness is reduced compared to uninfected

73 counterparts (Oliver et al., 2006). This interplay between reproductive strategy, context,
74 and evolutionary history illustrates the complexity and fluidity of symbiosis.

75 The facultative endosymbiosis between the social amoeba *Dictyostelium discoideum* and
76 *Burkholderia* bacteria provides a promising system for insight into symbiosis dynamics
77 (Brock et al., 2011; DiSalvo et al., 2015). *D. discoideum* is a soil dwelling amoeba with
78 an interesting life cycle involving unicellular and multicellular stages. During the
79 unicellular stage, amoebae consume bacteria by phagocytosis and divide. When prey
80 are scarce, amoebae aggregate by tens of thousands to form multicellular slugs that
81 move towards heat and light, seeking out a location to form fruiting bodies. These fruiting
82 bodies consist of a stalk of sacrificial dead cells which support a globular sorus
83 containing hardy spore cells (Kessin, 2001). When spores are dispersed, they germinate
84 into vegetative amoebae and the cycle continues. Processes employed by specialized
85 immune-like cells (sentinel cells) during slug migration and fruiting body formation
86 remove any remaining bacteria, typically producing bacteria-free sori (Brock et al., 2011;
87 Chen et al., 2007; Cosson and Lima, 2014).

88 However, some *D. discoideum* isolates harvested from the wild are infected with
89 *Burkholderia* symbionts (Brock et al., 2011). Infection persists in the lab throughout the
90 social cycle, where intracellular bacteria can be visualized within spores and sori
91 (DiSalvo et al., 2015). Infection can be terminated by treating hosts with antibiotics and
92 induced by exposing naïve hosts to *Burkholderia,* thereby allowing us to easily mix and
93 match partners and study subsequent fitness consequences (Brock et al., 2016a;
94 DiSalvo et al., 2015).

D. discoideum also has a meiotic sexual process, that occurs much less frequently than the asexual proliferation process of binary fission that amoebae go through every few hours (Bloomfield et al., 2010). The asexual binary fission process results in lineages that can be quite different, though all of the same species. Thus, a lineage that has acquired a bacterial endosymbiont can evolve to tolerate it independent of uninfected lineages at least for the thousands of generations before sexual recombination. Comparing such lineages can illuminate how natural selection operates in early stages of symbiosis.

The *Dictyostelium-Burkholderia* association is particularly compelling for studying the parasitism to mutualism continuum in endosymbiosis because the fitness consequences of infection are context dependent. Under standard laboratory growth conditions, *Burkholderia* infection is detrimental for hosts because it decreases slug migration and spore production (Brock et al., 2016a; DiSalvo et al., 2015). However, infection can be beneficial. Infection induces secondary carriage of edible bacteria (*Burkholderia* is typically inedible), which can increase host fitness in food scarce conditions when the carried food bacteria reseed new environments with a food source (Brock et al., 2011; DiSalvo et al., 2015). Additionally, infected hosts are less harmed by ethidium bromide exposure, possibly mediated by bacterial degradation of the toxin (Brock et al., 2016a).

We have identified three *Burkholderia* species associated with *Dictyostelium*: *B. agricolaris, B. hayleyella,* and *B. bonniea* (Brock et al., 2018). Most of our work has been conducted with the first two species, which differentially impact host fitness. Here we probe the interaction between host and symbiont genotypes (with regards to their association history) with infection outcomes. We use standard laboratory conditions in

which symbiont infection is shifted towards host detrimental outcomes. Since all three *Burkholderia* species can be cultured on Petri plates with standard media, none of them are entirely dependent on *Dictyostelium* for survival.

We find that *B. hayleyella* is most detrimental for *D. discoideum* hosts in general, but is most costly to those first exposed to it in the lab. We also document symbiont localization and morphological symptoms in hosts throughout development. Although both species of bacteria can be observed within phagocytic vacuoles, *B. hayleyella* infects more cells and damages fruiting body structures. These morphological aberrations are also less severe in native-*hayleyella* hosts, suggesting that native hosts have evolved mechanisms to withstand symbiont colonization.

RESULTS

Impact of B. agricola *and* B. hayleyella *on* D. discoideum *spore production*

Our first goal was to clarify how infection of *Dictyostelium* by *Burkholderia* symbionts differentially influences host fitness. We used 12 wild-collected clones, from each of three original conditions, uninfected with *Burkholderia,* infected with *B. agricolaris,* or infected with *B. hayleyella* (Table 1). We refer to these three types respectively as naïve hosts, native-agricolaris hosts, and native-hayleyella hosts. The word “host” always means *D. discoideum,* and sometimes refers to potential hosts not actually infected with *Burkholderia.* Amoebae, spores, cells, slugs, fruiting bodies, stalks, and sori refer only to *D. discoideum.*

We looked at *D. discoideum* spore viability and other measures under the following conditions: a) natural field state (naive, native-*agricolaris*, native-*hayleyella*), b) those

same hosts cured of *Burkholderia* (antibiotic treated), and c) condition after curing and re-infecting (with either *B. agicolaris* or *B. hayleyella*) (Figure 1). We quantified percent spore viability and number of spores produced (Figure 2, and Supplemental Tables S1-S2). We multiplied these two measures to get a single main measure of fitness, viable spores produced.

D. discoideum *fitness does not differ by wild* Burkholderia *infection status*

We found that infection status in the field did not affect total viable spore counts for naïve, native-*agricolaris*, or native-*hayleyella* hosts (Figure 2a and Table S1a) (linear mixed model (LMM), $\Delta AIC = -1.57$, $\chi^2 = 5.57$, DF = 2, $P = 0.062$). Thus, field-infected native hosts do not seem to suffer any net fitness costs from infection by this measure.

D. discoideum *fitness does not decrease with antibiotic treatment*

To make parallel comparisons when we newly infected *D. discoideum* with either of the two *Burkholderia* species, we first had to cure all hosts and be sure that curing in itself did not decrease fitness. We found that wild-collected hosts of our three categories did not experience lowered fitness after being cured with antibiotics (Figure 2a&b) (viable spore production: LMM, $\Delta AIC = 1.74$, $\chi^2 = 2.26$, DF = 2, $P = 0.32$, Table S1b). Antibiotic treatment actually increased viable spore production of native-*agricolaris* hosts compared to uncured native-*agricolaris* hosts (LMM, $\Delta AIC = -5.68$, $\chi^2 = 9.68$, DF = 2, $P = 0.008$; Figure 2a&b).

D. discoideum *fitness decreases with exposure to* Burkholderia

To test the effects of *Burkholderia* on all types of field-collected hosts, we compared viable spore production of cured *D. discoideum* hosts versus those same hosts artificially infected with *B. agricolaris* or *B. hayleyella* (Figure 2b, 2c, 2d). We found an overall effect on total spore viability with the addition of *Burkholderia* to all antibiotic-cured hosts (LMM, $\Delta AIC = -198.81$, $\chi^2 = 210.81$, DF = 6, $P \ll 0.001$). Addition of either *B. agricolaris* (Wald $t = -10.19$, DF = 96, $P \ll 0.001$) or *B. hayleyella* ($t = -13.58$, DF = 96, $P \ll 0.001$) to any of the cured hosts decreased their fitness (Figure 2; Table S2).

We then asked whether *D. discoideum* hosts are adapted to the *Burkholderia* species they carried in the field. Overall, the addition of *B. hayleyella* to *D. discoideum* led to significantly lower viable spore production than did the addition of *B. agricolaris* (Wald $t = -4.48$, DF = 96, $P \ll 0.001$) (Figure 2). We also tested for an interaction between native host status and which *Burkholderia* species was added. There was an interaction effect on total viable spore production (LMM, $\Delta AIC = -199.93$, $\chi^2 = 215.92$, DF = 8, $P \ll 0.001$) (Table S2e). To address specific adaptation, we performed separate tests for each *Burkholderia* species added. When *B. hayleyella* was added to the three cured hosts, native-*hayleyella* hosts had higher fitness than did either native-*agricolaris* or naïve hosts (both $P \ll 0.001$, Figure 2d; Table S2e). In contrast, native-*agricolaris* hosts did not have significantly higher fitness with the addition of *B. agricolaris* than either native-*hayleyella* or naïve hosts (both $P > 0.05$, (Figure 2c;Table S2e). However, there was a trend in the direction of native-*agricolaris* doing best (Figure 2c). These results indicate that native-*hayleyella* hosts are adapted to colonization by their field-acquired symbionts.

D. discoideum *morphology and* Burkholderia *infected state*

We next examined host morphology and symbiont localization at several stages of the *D. discoideum* life cycle. Using transmission electronic and confocal microscopy, we examined one *D. discoideum* clone for each host type outlined above and in Figure 1 (QS9 for the naïve, QS70 for the native-*agricolaris*, and QS11 for the native-*hayleyella* host). These were either in an uninfected state or infected with one representative of *B. agricolaris* (Ba70 from QS70) or of *B. hayleyella* (Bh11 from QS11).

***Food bacteria location inside* D. discoideum *uninfected with* Burkholderia**

D. discoideum morphology without *Burkholderia* in both naïve and cured native hosts, has vegetative cells that harbor no intracellular bacteria but contain many empty multilamellar bodies inside food vacuoles (Figure 3a and Supplemental figure S2). Confocal microscopy of vegetative amoebae from this same host set grown with GFP-labeled food bacteria (*K. pneumoniae*) contain little to no intracellular GFP, suggesting that they have digested food bacteria by the time of fixation (Figure 4a).

After bacterial food has been depleted, vegetative cells aggregate to form multicellular migratory slugs. In accordance with our observations that all bacteria were killed and digested by vegetative cells, we found no intact bacteria in naïve or cured native host slugs (Figure 5a). In addition, slug cells were in general compacted with electron dense materials and contained no food vacuoles or multi-lamellar bodies (Figure 5a).

Ultimately, slug cells differentiate into fruiting bodies consisting of dead stalk cells that support a sorus containing reproductive spore cells. In uninfected fruiting bodies, we did not detect bacteria in stalk cells or spores (Figure 6a and 7a). Instead spores were packed with electron dense materials with no food vacuoles or multi-lamellar bodies

(Figure 6a). Stalk cells showed plant cell-like characteristics, having a cellulosic cell wall and containing a single large vacuole (Figure 6a). Inside the large vacuole, there were some mitochondria and other cellular materials but no bacteria (Figure 6a). Taken together, these results suggest that the food bacterium we used*, Klebsiella pneumoniae* was efficiently cleared during the social cycle from amoebae uninfected by *Burkholderia* and the amoebae then aggregate and produce bacteria-free fruiting bodies.

Burkholderia *location inside* D. discoideum

When *D. discoideum* is infected with *Burkholderia,* we find it in amoebae, slug cells, spores, and stalk cells. Using confocal microscopy and RFP labeled *Burkholderia* strains, we were able to specifically identify high levels of *Burkholderia* inside host amoebae (Figure 4b,c). *B. hayleyella* was present in more of the amoebae than *B. agricolaris* was (Figure 4b,c). The higher number of *B. hayleyella* bacteria may account for the more detrimental fitness consequences it imposes (Figure 2).

The food bacterium *K. pneumoniae* labelled with GFP was occasionally observed in *Burkholderia* infected amoebae, particularly those infected by *B. agricolaris* (Figures 4b, 7b). This suggests that *Burkholderia* colonization may partially impede food digestion, thereby allowing co-colonization of secondary bacteria and contributing to the proto-farming phenotype (Brock et al., 2011; DiSalvo et al., 2015). However, confocal images demonstrate that *Burkholderia* is much more abundant in amoebae than is *K. pneumoniae*, allowing us to infer that the majority of intact intracellular bacteria observed via transmission electron microscopy (TEM) are most likely *Burkholderia* cells (Figures 3, 5, 6).

TEM of vegetative amoebae shows intact bacteria which we infer to be *Burkholderia* surrounded by multi-lamellar bodies and located inside what appear to be food vacuoles (Figure 3b,c). Their undamaged appearance suggests that they are resistant to phagocytic digestion. In addition to the presence of intact intracellular bacteria, we also observe empty multi-lamellar bodies inside food vacuoles of infected amoebae. This suggests that digestion is not completely arrested during colonization, an unsurprising finding given that hosts continue to grow and multiply. Interestingly, we did not find multi-lamellar bodies containing intact bacteria secreted into the extracellular environment, indicating that *Burkholderia* may not be expelled from host cells via multi-lamellar body excretion.

In *Burkholderia* infected hosts, intact bacteria are retained in food vacuoles throughout the transition to multicellular slugs, suggesting that bacteria stay within phagosomes throughout the aggregation stage (Figure 5b,c). Through TEM, we did not detect obvious morphological defects in infected slugs or differences between slugs infected with different *Burkholderia* species.

After fruiting body development, we find intracellular bacteria in both stalk and spore cells of *Burkholderia* infected hosts (Figure 6b,c). In infected stalk cells, intact bacteria reside in single large vacuoles inside the cellulosic cell wall (Figure 6b,c). In spore cells, bacteria remain within vacuoles (Figure 6b,c). We also observed bacterial cells outside spores but within the sorus, suggesting that bacteria can either travel extracellularly into the sorus or escape from spores after sorus formation (Figure 7b,c).

We did not observe strikingly altered morphologies for *B. agricolaris* infected spore and stalk cells (Figure 6b). However, the spore and stalk cells of naïve hosts infected with *B. hayleyella* appeared to be morphologically aberrant (Figure 6b). We found numerous broken spores and signs that bacterial cells were escaping from damaged spores (Figure 6b). In addition, the whole stalk structure was often collapsed and filled with bacteria (Figure 6b). No clear cellulosic cell wall was observed in stalk cells, suggesting that *B. hayleyella* colonization inhibits the normal development of stalk cells or results in their disruption in naïve hosts and native-*agricolaris* hosts.

In line with the visualized differences between the abundance of the two species of *Burkholderia* in amoebae, we find significantly more naïve spores infected with RFP labeled *B. hayleyella* (mean= 88.8%) than similarly labeled *B. agricolaris* (mean= 35.3%) ($F_{2,4}$ = 191.33, $P < 0.001$) (Figure 7d). These results indicate that the degree of fitness detriment imposed by *B. hayleyella* could be a result of bacterial density.

Burkholderia *impact on fruiting body morphology*

Since *Burkholderia* are found inside *D. discoideum,* it is no surprise they impact the morphology of fruiting bodies. In their field-collected state, the three clones carrying no *Burkholderia,* or *B. agricolaris* or *B. hayleyella* differed in both stalk height and stalk volume ($F_{2,27}$ = 42.6, $P \ll 0.001$, $F_{2,27}$ = 50.8, $P \ll 0.001$, Figure 8b, Table S3a). The main pattern is that both height and volume were significantly lower in native-*hayleyella* hosts (Fig. 8; Table S3a).

Native-*agricolaris* fruiting bodies were generally similar to the naïve host but taller than the native-*hayleyella* host ($P < 0.001$). In the native-*agricolaris* host, the spore masses

often slid down their stalks or the fruiting bodies fell over, though the stalks were not significantly taller than in the naïve host. If the spores fall off their stalks, they will not have the advantage of facilitated transport by a vector that they would have at the top of the stalk (smith et al., 2014).

There was also an overall difference in sorus diameter and sorus volume among the three hosts in their field state (Figure 8; $F_{2,236}$ = 25.4, $P \ll 0.001$, $F_{2,236}$ = 22.9, $P \ll 0.001$, Table S4a). Compared to the naïve hosts, both the native-*agricolaris* and native-*hayleyella* hosts had smaller sorus sizes (both $P < 0.001$) but were not different from each other.

Curing with antibiotics caused no significant change in any stalk or spore measurements (Figure 8a, b, c; Table S3b, S4b). However, when *Burkholderia* bacteria were added to the cured hosts, we saw species-specific effects on morphology (Figure 8; Table S3c-d, S4c-d). Overall, the addition of *B. agricolaris* changed stalk height ($F_{1,58}$ = 54.9, $P \ll 0.001$) significantly increasing it in two of the hosts (Table S3c). Stalk volume was not affected. Addition of *B. agricolaris* also significantly changed both sorus diameter and sorus volume ($F_{2,240}$ = 25.4, $P \ll 0.001$, $F_{2,240}$ = 21.2, $P \ll 0.001$). Native-*hayleyella* hosts had larger sori when infected with *B. agricolaris* (Figure 8; Table S4c).

The addition of *B. hayleyella* decreased both stalk height (Figure 8; $F_{1,58}$ = 366.4, $P \ll 0.001$) and stalk volume ($F_{1,58}$ = 120.2, $P \ll 0.001$, Table S3d) significantly in all three hosts. Addition of *B. hayleyella* also affected sorus diameter ($F_{2,240}$ = 10.1, $P \ll 0.001$) and volume ($F_{2,240}$ = 11.3, $P \ll 0.001$) with a significant specific effect of smaller sori in the naïve host (Figure 8; Table S4d).

There were interaction effects between the amoebae hosts' native infection status and the species of *Burkholderia* added for all traits: stalk height ($F_{4,81}$ = 4.9, P = 0.0015), stalk volume ($F_{4,81}$ = 7.4, $P \ll 0.001$; Figure 8; Table S3e), sorus diameter ($F_{4,719}$ = 5.3, P = 0.0004) and sorus volume ($F_{4,719}$ = 4.5, P = 0.0014; Figure 8; Table S4e). However, these interactions were matters of degree of change and did not involve sign changes: for all four measurements, fruiting bodies with *B. agricolaris* were taller than those with *B. hayleyella* (Figure 8; Tables S3e, S4e).

DISCUSSION

Here, we characterized *D. discoideum* infection by two symbiotic *Burkholderia* species, *B. hayleyella* and *B. agricolaris* (Brock et al., 2018). We looked at their impact on *D. discoideum* by comparing wild type, cured, and re-infected hosts. We assessed fitness measured as production of viable spores, and also evaluated morphological changes in amoebae, slugs, and fruiting bodies with numerical and microscopic data. We found that both *Burkholderia* species are a burden to *D. discoideum* under our experimental conditions. However, wild collected hosts did not differ in viable spore production according to whether or not they carried either species of *Burkholderia.* Even so, *D. discoideum* with their field-collected state of infection did differ in fruiting body dimensions, with uninfected hosts generally having taller, larger stalks, and larger sori. What explains the differences from experimental infection is unclear. Infection in the wild may be at a lower level than we used experimentally or may have initiated at a lower level that slowly amplified over time, allowing host acclimation to the metabolic costs of infection.

313 Once *D. discoideum* hosts are cured with antibiotics, so all comparisons can start from
314 the same baseline, we found that there were few within treatment differences according
315 to host type. The only exception to this is that native-*hayleyella* hosts produced more
316 viable spores than did naïve or native-*agricolaris* hosts. This fitness difference is an
317 indication of co-adaptation.

318 Perhaps the general lack of difference among host types with infection is due to sex,
319 which not only recombines genes, but also exposes new clones horizontally to
320 endosymbionts like *Burkholderia. Dictyostelium* recombination rates are high in natural
321 populations (Flowers et al., 2010). The differences reported here among clones of *D.*
322 *discoideum* indicate that the sexual stage has not particularly disrupted specific co-
323 adaptation in clones infected with *B. hayleyella.* Thus reproduction by binary fission and
324 vertical transmission of *B. hayleyella* is likely to occur much more often than sexual
325 reproduction which would result in horizontal transmission.

326 Our previous work demonstrated that *Burkholderia* infections have contextually
327 dependent costs and benefits for their hosts. In food abundant conditions (which we
328 used here) *Burkholderia* infections are generally detrimental to host fitness (Brock et al
329 2011). However, when dispersed to food scarce conditions, *Burkholderia* infected hosts
330 are able to transport food bacteria with them which restocks their food source and results
331 in higher host fitness ("farming"). These fitness outcomes may result from the ongoing
332 power play underlying long-term symbiosis.

333 A compelling question is what mediates this tolerance to *B. hayleyella* infection in its
334 native host? It is possible that native hosts better inhibit infection events or better control

335 intracellular replication of symbiont cells. The percent of spore cells infected in the
336 population post symbiont exposure are not significantly different between a native-
337 *hayleyella* host and naïve hosts, which does not support the idea that infection events
338 are inhibited. However, our TEM analysis qualitatively points to the idea that after
339 infection, intracellular replication rates may differ between naïve and native-*hayleyella*
340 hosts. TEM images of native and naïve *B. hayleyella* hosts consistently suggest a higher
341 load of intracellular bacterial cells at each stage. For instance, naïve *B. hayleyella* hosts
342 produce paltry stalks that appear overwhelmed by bacterial cells and infected spore cells
343 that look on the verge of deteriorating. Neither of these extreme states were observed in
344 the native host. This could be mediated by host countermeasures that control
345 intracellular symbiont growth or disarm potential toxic symbiont byproducts.

346 Compared to *B. hayleyella, B. agricolaris* infections result in more modest (and
347 statistically insignificant) drops in *D. discoideum* viable spore production for naïve and
348 cured re-infected native-*agricolaris* hosts. In line with *B. agricolaris* being less invasive to
349 the host population it also appears in only about a quarter of spores after exposure.
350 Despite this, it is maintained in infected population of cells throughout the social stage
351 and over multiple social cycles (DiSalvo et al., 2015).

352 Earlier studies have identified other differences between naïve *D. discoideum* and those
353 carrying *B. hayleyella* (Brock et al., 2013, 2016b; Stallforth et al., 2013). *D. discoideum*
354 hosts carrying *B. hayleyella* harmed symbiont-free *D. discoideum* clones, causing them
355 to lose in social competition (Brock et al. 2013). In another study we compared cured
356 and uncured clones of *B. hayleyella* and found that slugs from uncured clones move less
357 far across a Petri plate, a cost of infection (Brock et al. 2015). Interestingly, in the current

study we detected no visible differences between cured and uncured slug cells. The recent discovery of sentinel cells as innate immune cells (Chen et al. 2007) made us wonder about how they fare with *Burkholderia* infected hosts. We found that *D. discoideum* hosts with *Burkholderia* do not produce as many sentinel cells but even so seem as resistant to toxins as uninfected lines with normal levels of sentinel cells (Brock et al., 2016).

Another interesting recent result on the interaction between *D. discoideum* and bacteria involves the role of the lectin discoidin 1 (Dinh et al., 2018). Clones infected with *Burkholderia* produced much greater quantities of lectins early in the social stage compared to uninfected clones. These lectins coated the food bacterium *K. pneumoniae* allowing it to avoid digestion. This is undoubtedly just a beginning, though a fascinating one, in our understanding of how *Burkholderia* take over *D. discoideum* cellular machinery to change relationships with bacteria.

D. discoideum is already a popular system for examining the molecular mechanisms of bacterial pathogenesis for a variety of important pathogens (Cosson and Soldati, 2008). However, the *D. discoideum-Burkholderia* system holds unique potential for studying eukaryote-bacterial associations. Given its natural occurrence, we can perform long-term ecological surveys, easily isolate new host-symbiont pairs, investigate naturally derived vs newly induced associations using a variety of partner pairing, and we can do experiments in evolution allowing each partner to evolve together or separately.

In addition, this work demonstrates several properties of this interaction that are distinct from other bacterial associations, possibly owing to its natural prevalence. For instance,

Bordetella bronchiseptica can intracellularly infect vegetative *D. discoideum* amoebae and persist in sorus contents. However, in contrast to *Burkholderia, B. bronchiseptica* is localized extracellularly in sori rather than inside spore cells (Taylor-Mulneix et al., 2017). Infections of *D. discoideum* with other intracellular pathogens such as *Legionella pneumophila* often produce secreted multi-lamellar bodies which harbor the bacterial pathogen (Denoncourt et al., 2014; Paquet and Charette, 2016), while in this study we found no evidence of *Burkholderia* excreted in multi-lamellar bodies. In addition, many bacteria resistant to amoebae are found packaged in multi-lamellar bodies, a process speculated to enhance their resistance to environmental stress. Thus, *Burkholderia* symbionts are most likely employing alternative tactics to not only evade digestion, but to also evade expulsion.

Burkholderia is important in another model symbiosis system, that with the bean bug, *Riptortus pedestris* (Takeshita and Kikuchi, 2017). These bugs acquire *Burkholderia insecticola* horizontally. They reside in the bean bug gut where they are presumably active in nutrition. They are a good model because it is a facultative symbiosis and both partners can be cultured independently.

This is a new age of symbiosis studies where we can apply Koch's principles of curing, re-infecting, and looking for evidence of disease. We can use genomics, experimental evolution and many other methods to ever more systems. In time, *B. hayleyella* and *B. agricolaris* in *D. discoideum* may be added to the classic symbioses of squid-vibrio, aphid-*Buchnera*, tsetse fly-*Wiggelsworthia,* legume-*Rhizobia* and more (Bennett and Moran, 2015; Bing et al., 2017; Koehler et al., 2018; Werner et al., 2015).

MATERIALS AND METHODS

D. discoideum strains and culture conditions

We collected *D. discoideum* isolates from the field that were uninfected, or infected with either *B. agricolaris*, or *B. hayleyella.* Table 1 describes host clone sets, location collected, and infection status. We used host sets 1-4 for the spore fitness assays and set 1 for all other experiments. We used *Klebsiella pneumoniae* obtained from the Dicty Stock Center (http://dictybase.org/StockCenter/StockCenter.html) as our food bacterium for *D. discoideum.* We grew all *D. discoideum* from spores on SM/5 agar plates (2 g glucose, 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$, 1.9 g KH_2PO4, 1 g K_2HPO_4 and 15.5 g agar per liter) supplemented with *K. pneumoniae* at room temperature (21°C).

Symbiotic bacterial strains

We used *D. discoideum*-associated *Burkholderia* previously isolated and sequenced to verify closest 16S identity (Brock et al., 2011; DiSalvo et al., 2015). *B. agricolaris* and *B. hayleyella* strains were isolated from QS70, QS159, QS161, and NC21, and QS11, QS23, QS22, and QS21 *D. discoideum* hosts respectively.

Removal of symbiont from native *D. discoideum* hosts

We generated symbiont-free native host clones by tetracycline, or by ampicillin-streptomycin, treatment as previously described (Brock et al., 2011; DiSalvo et al., 2015). We confirmed loss of infection status using the spot test assay and PCR analysis of *Burkholderia*, and *K. pneumoniae* in *D. discoideum* sori as previously described.

Lab Infections

We collected stationary phase bacteria in starvation buffer from bacteria grown on SM/5 plates. We determined the bacterial absorbance (A_{600}) using a BioPhotometer (Eppendorf, NY) and set all suspensions to optical density (OD_{600} 1.5). For experiments using lab-infected lines, we mixed the specified *Burkholderia* species at 5% and *K. pneumoniae* at 95% volume and plated *D. discoideum* spores (as indicated) with 200ul of the bacterial mixture on SM/5 plates.

Spot test assay

We verified infection status by spot test assay as previously described (Brock et al., 2011). Briefly, we transferred sorus contents from individual *D. discoideum* fruiting bodies to SM/5 agar plates using a 10µl filter pipet tip. We incubated at 21°C for one week and checked for bacterial growth as an indication of infection.

Fitness assay

We analyzed spore production and viability as a proxy for amoeba fitness using four sets of *D. discoideum* clones (Table 1). We tested three conditions: uninfected (naïve, cured naïve, cured native-*agricolaris*, and cured native-*hayleyella*), *B. agricolaris* infected (native-*agricolaris*, and naïve, native-*agricolaris*, and native-*hayleyella* first cured then infected with *B. agricolaris*) and B. hayleyella infected (native-*hayleyella*, and naïve, native-*agricolaris*, and native-*hayleyella* first cured then infected with *B. hayleyella*) across three temporal replicates.

443 To set up each assay, we plated 2 × 10^5 spores of each clone in each condition (with
444 lab infected lines being plated on *Burkholderia-Klebsiella* mixtures as described) onto
445 SM/5 agar plates in duplicate. All clones formed fruiting bodies by 3 days, so we
446 performed data collection five days after fruiting. We used the first plate to ascertain
447 total spore production as previously described (DiSalvo et al., 2015). Briefly, spores
448 were collected by washing plates with starvation buffer supplemented with 0.01% NP-
449 40 alternative (Calbiochem). We counted spore dilutions on a hemocytometer using a
450 light microscope and determined total spores according to total volume collected and
451 dilution factor. To determine the proportion of viable spores we collected spores into
452 starvation buffer only and determined spore density as above. We diluted suspensions
453 to 10^4 and spread 100 spores over ten 100 × 15 mm^2 Sm/5 agar plates supplemented
454 with 200 µl *K. pneumoniae* in starvation buffer (absorbance, A_{600} 1.5). After 2 days the
455 percentage of viable spores was determined by counting plaques formed on bacterial
456 lawns.

457 **Transmission electron microscopy**

458 We prepared amoebae by plating 2 × 10^5 spores (with *Klebsiella* for uninfected or native-
459 infected and for the indicated *Burkholderia* mixture for lab-infected). We harvested log-
460 phase vegetative cells approximately 36hrs after plating and fruiting bodies 4 days after
461 plating. To prepare migrating slugs, we mixed 200 µL of centrifuge-concentrated *K.*
462 *pneumoniae* (absorbance, A_{600} 75) with 5 x10^6 spores and plated the mixture in a
463 straight line across a starving agar plate, which was then wrapped in aluminum foil with a
464 small hole opposite the spore line. We incubated plates under a direct light and allowed
465 slugs to migrate for about 80 hours before processing. We processed all stages by first

466 adding fix solution (2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc.,
467 Warrington, PA) in 100 mM cacodylate buffer, pH 7.2), followed by low melting agarose,
468 over the plates to keep structures intact.

469 We fixed samples for 1-3 hr at room temperature then washed with cacodylate buffer
470 and postfixed in 1% osmium tetroxide (Polysciences Inc.) for 1 hr. We then rinsed
471 samples extensively in dH_2O prior to *en bloc* staining with 1% aqueous uranyl acetate
472 (Ted Pella Inc., Redding, CA) for 1 hr. Following several rinses in dH2O, we dehydrated
473 samples in a graded series of ethanol and embedded them in Eponate 12 resin (Ted
474 Pella Inc.). Sections of 95 nm were cut with a Leica Ultracut UCT ultramicrotome (Leica
475 Microsystems Inc., Bannockburn, IL), stained with uranyl acetate and lead citrate, and
476 viewed on a JEOL 1200 EX transmission electron microscope (JEOL USA Inc.,
477 Peabody, MA) equipped with an AMT 8 megapixel digital camera (Advanced Microscopy
478 Techniques, Woburn, MA).

479 **Confocal microscopy**

480 We constructed RFP labeled versions of *B. agricolaris* (from QS70) and *B. hayleyella*
481 (from QS11) by performing triparental mating procedures with the *E. coli* helper strain
482 E1354 (pTNS3-asdEc) and the *E coli* donor strain E2072 with pmini-Tn7-gat-P1-rfp and
483 confirmed glyphosate resistant RFP positive *Burkholderia* conjugants using *Burkholderia*
484 specific PCR as previously described (DiSalvo et al., 2015; Norris et al., 2009; Su et al.,
485 2014). We constructed a GFP labeled version of *K. pneumoniae* using a triparental
486 mating strategy with the donor *E. coli* donor strain WM3064 containing pmini-Tn7-KS-
487 GFP and the *E. coli* helper strain E1354 helper pUXBF13 as previously described

(Kikuchi and Fukatsu, 2014). We confirmed kanamycin resistant GFP positive recipient cells by 16s rRNA gene sequencing.

Using *Burkholderia*-RFP and *Klebsiella*-GFP we infected set 1 hosts (using cured native hosts) as previously described. Control samples were plated with *K. pneumoniae*-GFP only. We harvested log-phase amoebae approximately 36 hrs after plating by flooding plates with 5ml SorMC and washing 3x in PBS to remove residual bacteria. We set amoebae to 1 x 10^6 cells/ml and placed 200µl onto #1.5 glass coverslips for 15min to allow them to adhere before fixing in 4% formaldehyde for 10min. We then washed with PBS, permeabilized with 0.5% triton-X, and stained with Alexa Fluor® 680 phalloidin (lifetechnologies) for 30 min before mounting in Prolong® Diamond antifade mountant (lifetechnologies). We prepared spores four days after plating by collecting sori into starving buffer with 1% calcofluor white and spreading the solution on a glass bottom culture dish under a 2% agarose overly.

We collected images using a Nikon A1Si Laser Scanning confocal microscope with a CFI Plan Apo VC Oil 1.4 NA 100X objective and Nikon Elements software or an Olympus Fluoview FV1000 confocal microscope using Plan Apo Oil 1.4NA 60X objective and Olympus software. Z-sections were taken every 0.5 microns with an average of 2 at 1024 x 1024 resolution pixels or 600 x 600 pixels. We excited RFP using the 561 laser, GFP with the 488 laser, Calcoflour-white with the 408 laser, and Alexa Fluor 680 Phalloidin with the 640 laser. We created composite images in FIJI.

Infectivity Quantification

We quantified the population of *Burkholderia*-RFP infected spores for the set 1 clones using the BD accuri C6 flow cytometer. We plated spores in duplicate as described in the confocal microscopy section. Four days after plating, we resuspended 3 sori from each plate into 500ul of starving buffer with 0.01% NP-40 alternative. We ran 100 µl of each vortexed sample through the flow cytometer. We used non-fluorescent controls to establish an accurate gating between fluorescent and non-fluorescent boundaries. We measured and averaged duplicates for a total of 6 temporal replicates.

Morphometrics

We quantified fruiting body size and shape for each clone in each condition using set 1 clones. We plated the clones as described under fitness assays. Five days after fruiting, we carefully cut and removed a thin strip of agar approximately 5 mm wide from the central area of an experimental plate and laid it on its side in a Petri plate. We placed dampened Kimwipes around the agar slice to prevent desiccation. We used a Leica EC3 scope with the LASD core package LAS V4.1) to collect data. Fruiting body images were taken randomly along with graticule images for calibration. We took six measurements of each fruiting body: sorus width, sorus length, stalk height and the width of the stalk at its base, midpoint and at the top just below the sorus (Buttery et al., 2009). Stalk height was measured from the base of stalk to the tip of the sorus. We calculated sorus volume applying the formula for the volume of a sphere using diameter, $V = 1/6\ \pi\ d^3$. We calculated stalk volume using the formula of a cylinder, $V= \pi\ r^2\ h$, where height (h) is the stalk height and radius (r) is half the mean of the three stalk width measurements. We measured about 80 sori and 20 stalks for each clone for each condition.

Statistical analyses

All analyses were done in R. For fitness assays, we tested the effect of antibiotic treatment using random-slope linear mixed models (LMM) on those *D. discoideum* hosts not reinfected with *Burkholderia*. Our models included either spore numbers or proportion of viability as the response variable, host as random effect, and antibiotic treatment as a fixed effect. We similarly tested the effects of *Burkholderia* infection in the field and in the lab using random-slope LMMs on data from hosts cured with antibiotics. Our models included spore numbers or proportion viability as response variable, host as random effect, lab or field-infection status and *Burkholderia* type as fixed effects, as well as an interaction between field-infection status and *Burkholderia* infection. For all LMMs, we fitted models and assessed model fit with likelihood ratio tests executed with the lme4 package (Bates et al., 2015) in the R environment (v. 3.3.3, R Core Team 2017). We tested the significance of fixed effects with Wald tests using the *t* distribution, which we executed with the packager lmerTest (Kuznetsova et al., 2017). These tests use (Satterthwaite, 1946) approximation for denominator degrees of freedom to calculate *p*-values. Finally, for all *post hoc* multiple comparisons, we performed pairwise contrasts of least-square means with a multivariate *t* distribution adjustment as implemented with the package lsmeans (Lenth, 2016).

For morphometric analyses, we also tested the effect of antibiotic treatment on those hosts not re-infected with *Burkholderia*. We used a 2-way analysis of variance (ANOVA) with one of our four morphological measurements as the response variable and both *Burkholderia* colonization and antibiotic treatment as fixed effects. Similarly, we tested the effects of *Burkholderia* colonization from the field and *Burkholderia* infection in the

lab on the amoeba hosts with 2-way ANOVAs on amoebae cured with antibiotics. Again, one of the four morphological measurements was the response variable with field-colonization status, *Burkholderia* infection, and an interaction between them as fixed effects. For all ANOVAs, when appropriate, we performed *post hoc* Tukey HSD tests for multiple comparisons. Sorus width data were square-root transformed as $\sqrt{x+2}$ and sorus volume data were $\log_e$-transformed to meet test assumptions of normally distributed residuals.

We analyzed *Burkholderia* infectivity in the spore population with a 2-way ANOVA, followed by a Tukey HSD for multiple comparisons. We treated *Burkholderia* species and *D. discoideum* host identity as fixed effects.

Contributions

Conception and planning: DAB, SD, DCQ, LS, JES

Experiments: DAB, SD, JM, LS

Manuscript: SD, DCQ, JES

Statistical analyses: SD, KG, DCQ

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Conflicts of interest

The authors declare no conflicts of interest.

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718

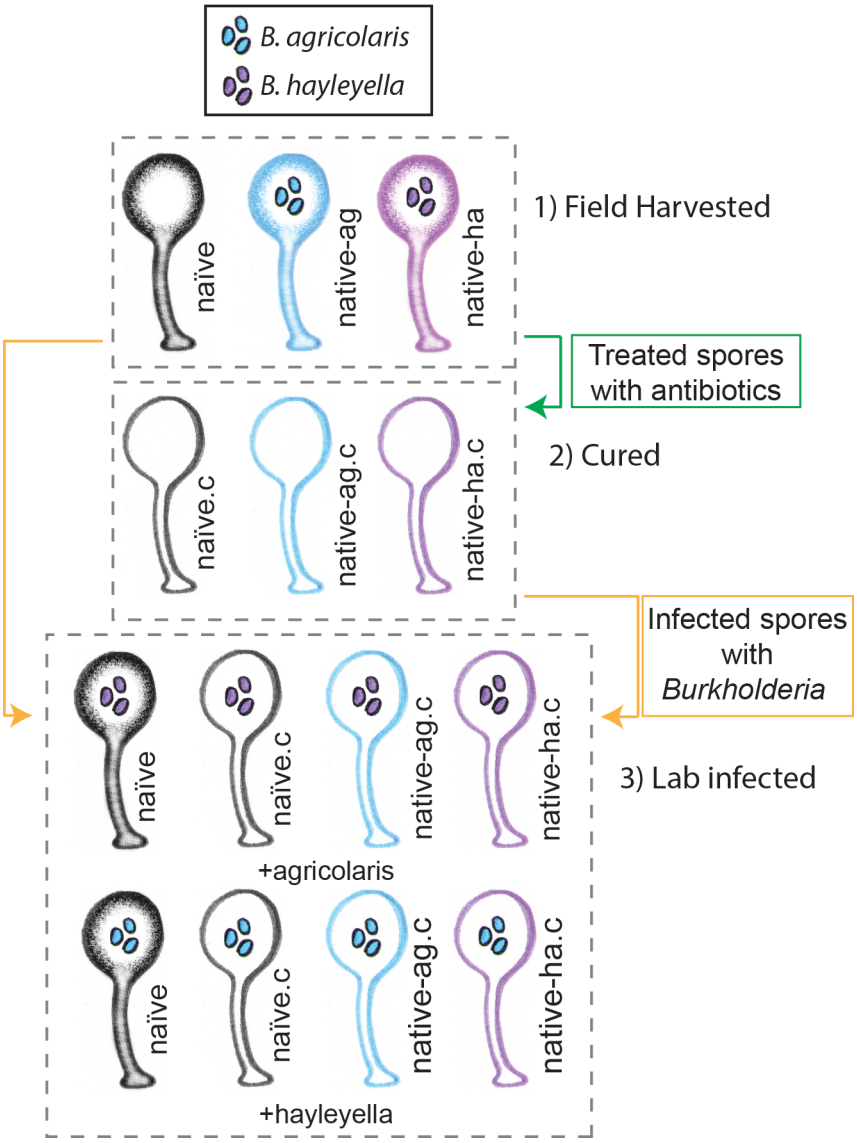
719 **Tables**

Set	Clone	Status	*Burkholderia*	Location Collected	GPS coordinates
1	QS9	Naïve	None	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	QS70	Native	*B. agricolaris*	Texas- Houston Arboretum	N 29° 46', W 95° 27'
	QS11	Native	*B. hayleyella*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
2	QS18	Naïve	None	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	QS159	Native	*B. agricolaris*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	QS23	Native	*B. hayleyella*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
3	QS17	Naïve	None	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	QS161	Native	*B. agricolaris*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	QS22	Native	*B. hayleyella*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
4	QS6	Naïve	None	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'
	NC21	Native	*B. agricolaris*	North Carolina-Linville Falls	N 35° 57', W 81° 57'
	QS21	Native	*B. hayleyella*	Virginia-Mt Lake Biological Station	N 37° 21', W 80° 31'

720

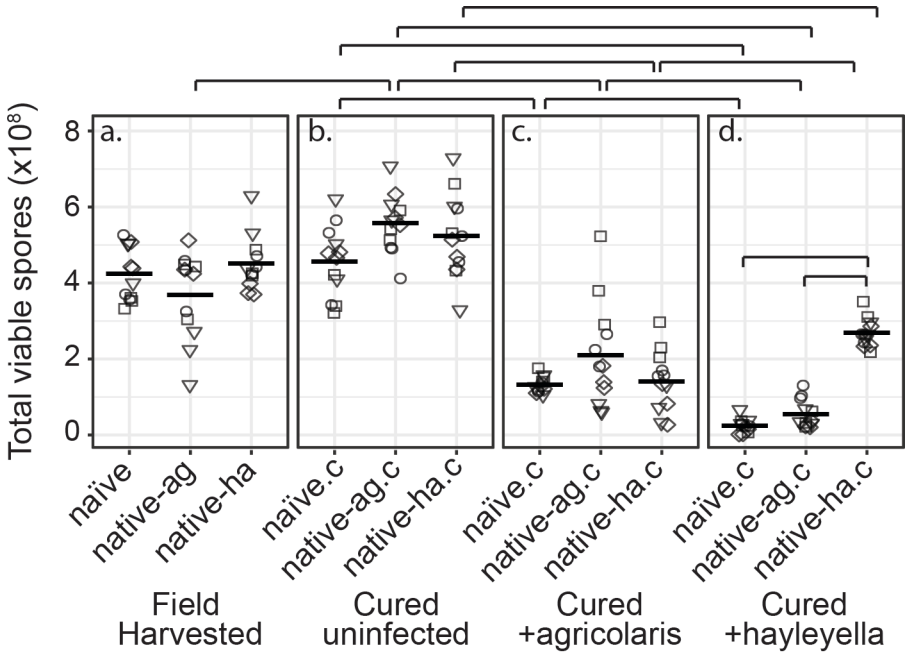
721 **Table 1. *Dictyostelium discoideum* clones used for this study.** Clones are divided
722 into specific sets each with naive, native-ag, and native-ha field-collected counterparts.
723 They were collected from Virginia, North Carolina, and Texas as indicated.

724 **Figures & Figure Legends**



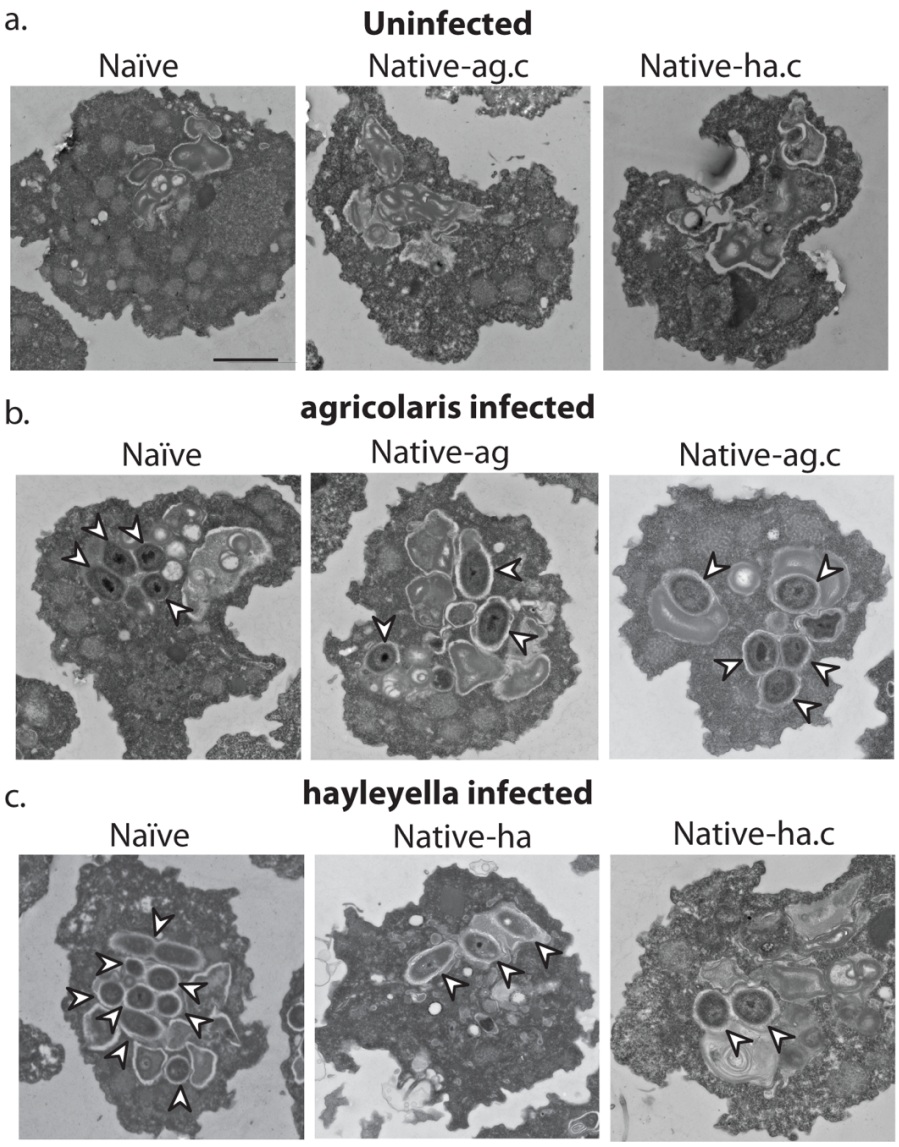
725

726 **Figure 1. Illustration of host-symbiont pairs used throughout the study.** *D.*
727 *discoideum* clones were originally harvested from the wild in three different states:
728 uninfected (indicated as naïve), or naturally infected with *B. agricolaris* or *B. hayleyalla*
729 (indicated as native-ag, and native-ha respectively). Clones were treated with antibiotics
730 to eliminate symbionts and are indicated with a ".c". Clones were subsequently exposed
731 to *Burkholderia* to initiate new infections. Thus, experimental types include 1) Field
732 harvested, 2) cured, and 3) lab infected hosts.



733

734 **Figure 2. *Burkholderia* Infections Differentially Alter Spore Viability According to**
735 ***Burkholderia* Species and Host Background.** Total viable spores were determined for
736 naïve and native hosts in their field harvested (a), cured (b), *B. agricolaris* lab-infected
737 (c), and *B. hayleyella* lab-infected state (d). Four clones were measured for each type
738 with 3 replicates for each (squares, triangles, circles, and diamonds represent set 1-4
739 clones respectively). Spore viability for wild harvested *B. agricolaris* and *B. hayleyella*
740 host clones is higher than their cured-re-infected counterparts. Notably, spores from
741 infected *B. agricolaris* and *B. hayleyella* native hosts (either naturally infected or cured
742 and re-infected with their original *Burkholderia*) have a higher fitness than *Burkholderia*
743 infected non-native counterparts. Bars represent significant differences ($p<0.05$, and as
744 indicated in supplemental tables).



745

746 **Figure 3. Bacterial cells are found within *Burkholderia* exposed vegetative**
747 **amoebae.** Transmission electron micrographs of vegetative amoebae show naïve and
748 cured native amoebae with intracellular morphologies suggestive of active bacterial
749 digestion with no evidence of intact intracellular bacteria (a). In contrast, bacterial cells
750 can be found within *B. agricolaris* (b) and *B. hayleyella* (c) infected hosts. Arrows point to
751 bacterial cells. More bacteria are observed in the *B. hayleyella* infected naïve host than
752 in field harvested native-*hayleyella* and cured and re-infected native-*hayleyella* hosts (c).
753 Bacterial cells appear to be within vacuole-like compartments. Scale bar (applicable to
754 all): 2um.

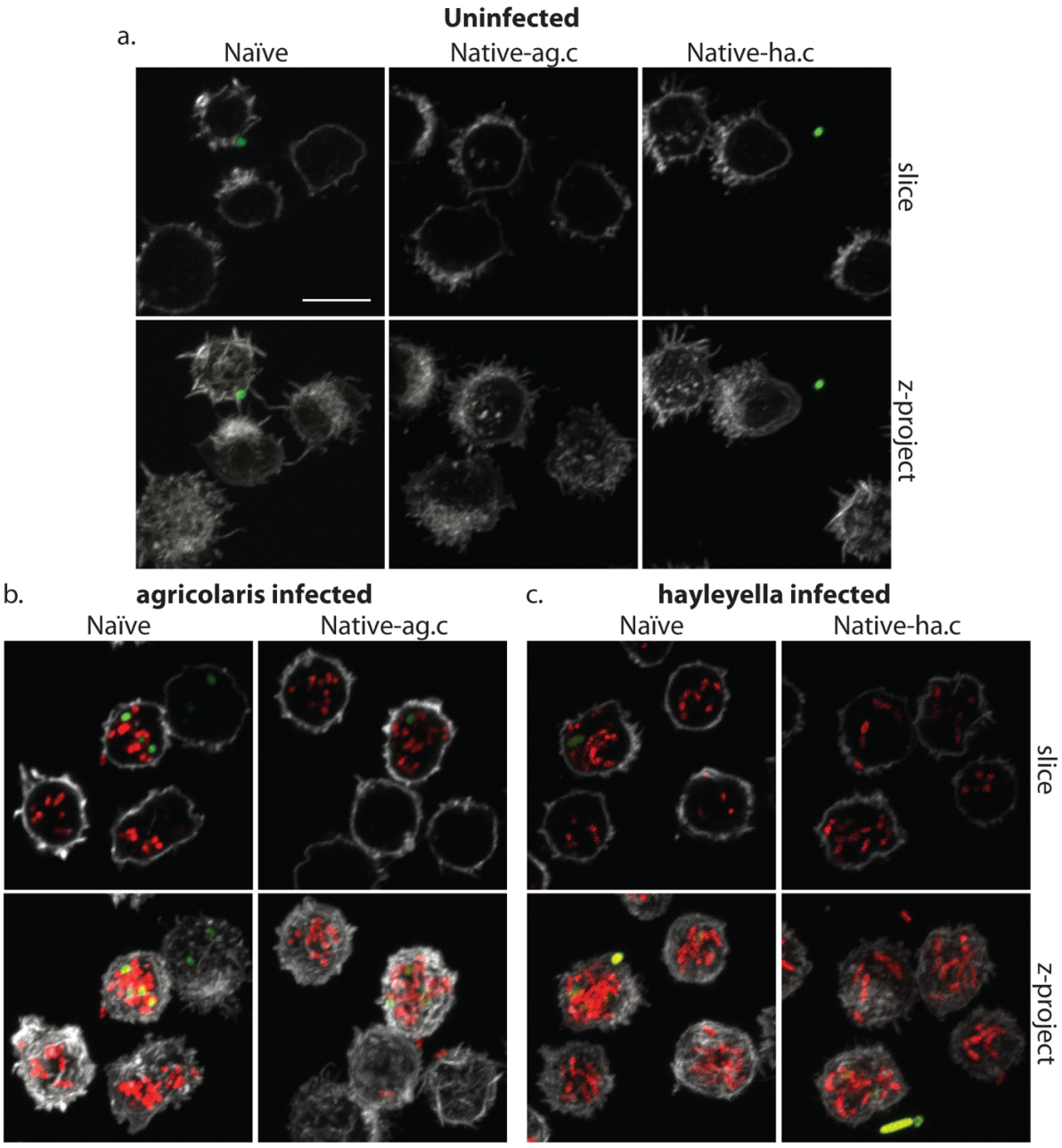


Figure 4. *Burkholderia* is found abundantly in colonized vegetative amoebae. Confocal imaging of fixed and stained vegetative amoebas show little to no intracellular bacteria in uninfected clones (a). However, abundant *Burkholderia* (*Burkholderia-RFP* shown in red) is found in *B. agricolaris* (b) and *B. hayleyella* (c) infected hosts. Occasional intracellular food bacteria (*Klebsiella*-GFP shown in green) is seen in *B. agricolaris* hosts (c). Spore coats are stained with phalloidin shown in grey. Scale bar 10um.

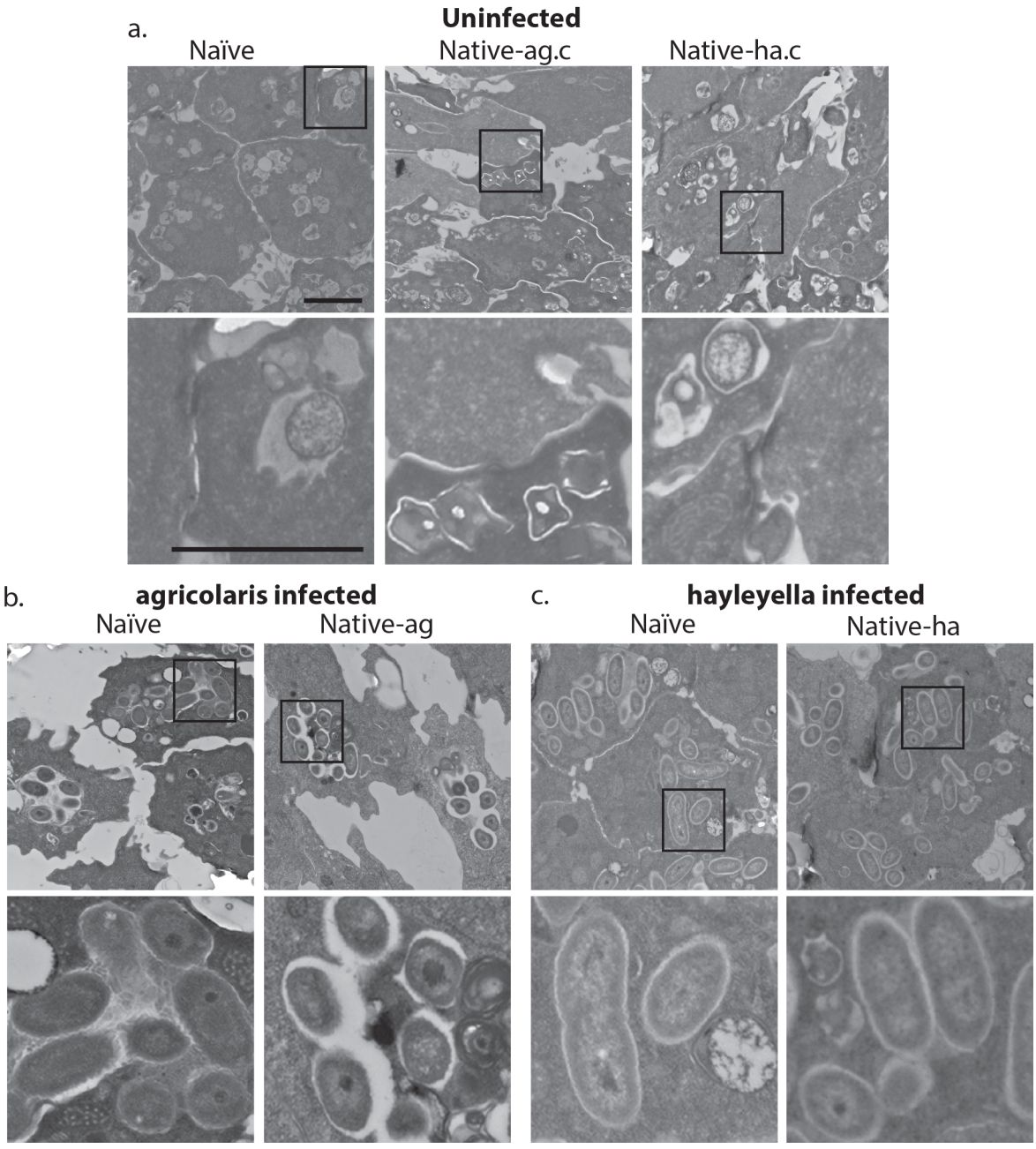


Figure 5. Intracellular bacteria are retained in naïve migrating slugs exposed to *Burkholderia* and in native *Burkholderia* hosts. Transmission electron micrographs of uninfected (a) show closely packed amoebae with internal structures reminiscent of previous bacterial digestion but without evidence of intact internal bacteria. In contrast, *B. agricolaris* (b) and *B. hayleyella* (c) infected slugs retain intracellular bacteria. Bottom panels represent magnified versions (see box) of upper panels. Scale bar (applicable to all panels in row) 2um.

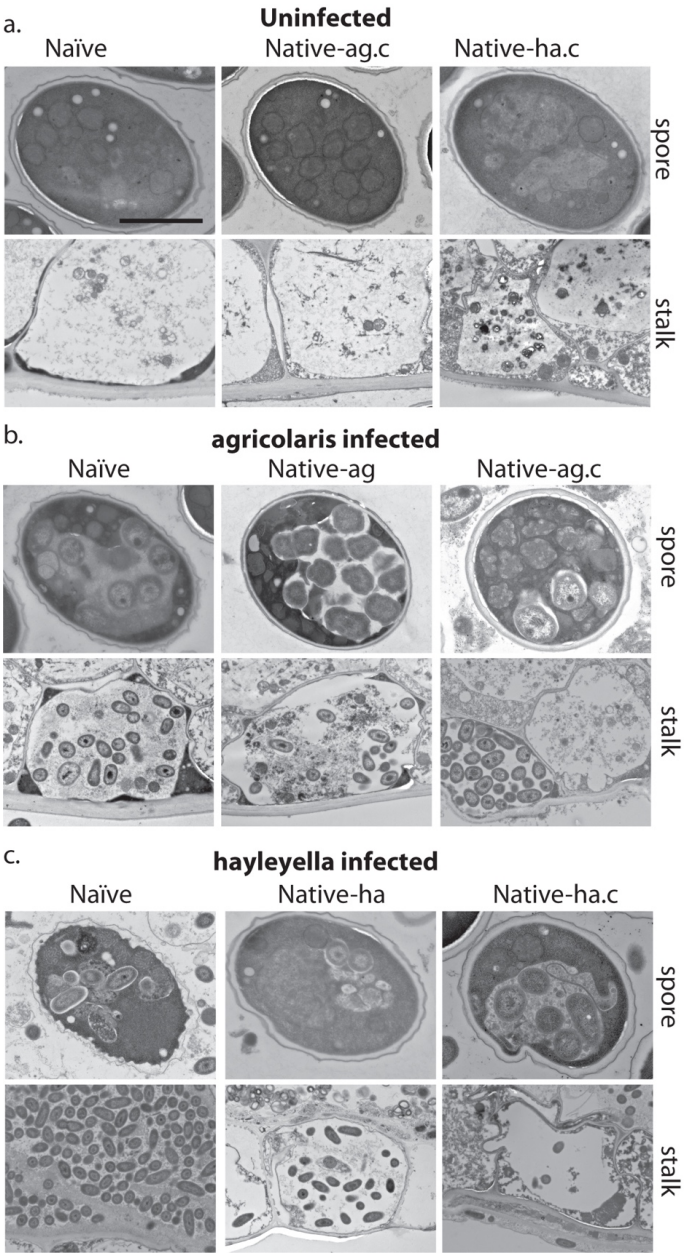
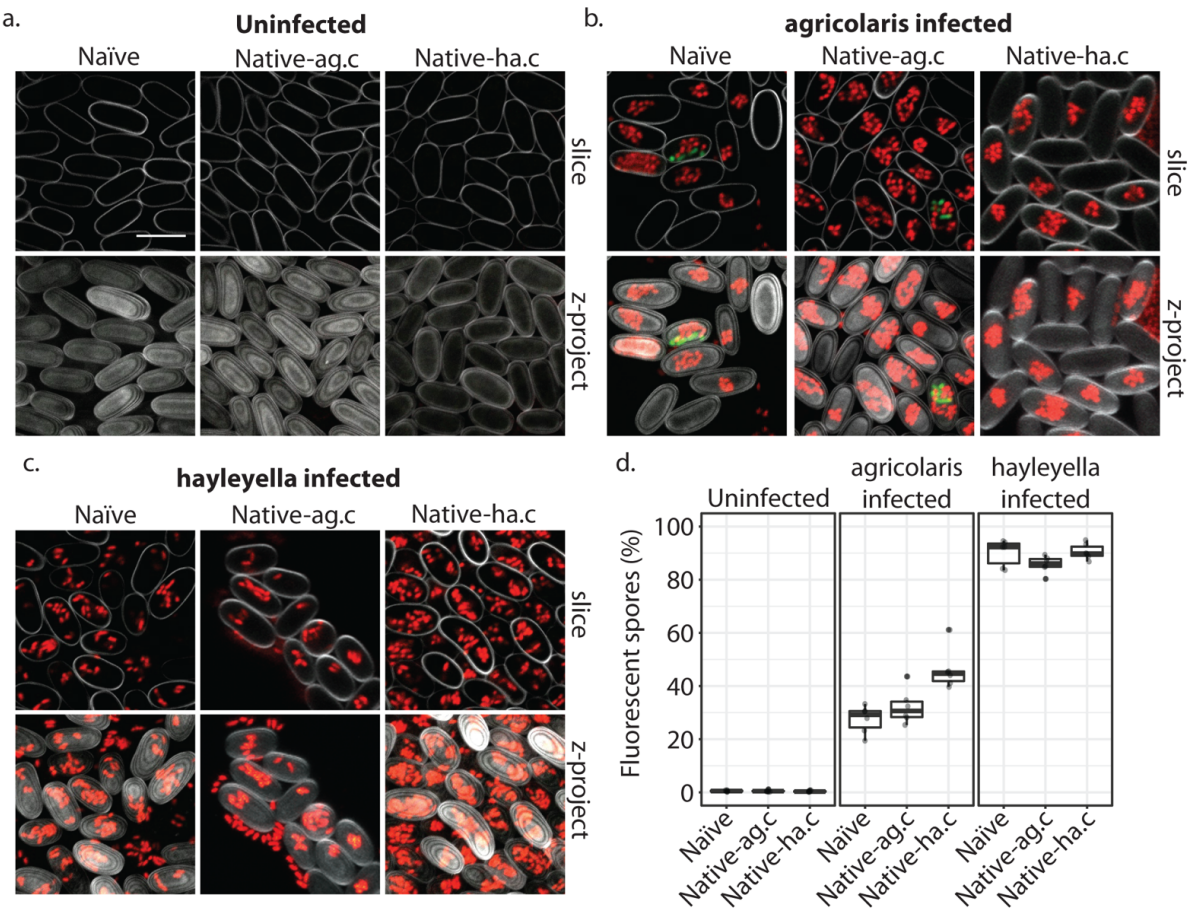


Figure 6. Bacterial cells are retained in spore and stalk cells from *Burkholderia*-exposed hosts. As visualized through transmission electron microscopy, (a) uninfected hosts form sturdy spores and stalk cells with no detectable bacteria. Spores and stalk cells retain intracellular bacteria in B. *agricolaris* (b) and B. *hayleyella* (c) hosts. Naïve *B. agricolaris* hosts appear structurally similar to uninfected cells while naïve *B. hayleyella* hosts have compromised spore coats and collapsed stalk structures filled with bacteria. Scale bar: 2um.



779

780 **Figure 7. *Burkholderia* is retained in the sori of developed *D. discoideum* hosts**
781 **and the percent of *Burkholderia* positive spores differs according to *Burkholderia***
782 **species.** Confocal images show no intra- or extracellular bacteria in uninfected spores
783 (a). Abundant *Burkholderia* is seen in *B. agricolaris* (b) and *B. hayleyella* (c) hosts, with
784 more infected spores seen for *B. hayleyella* (c). Detection of fluorescent spores
785 (indicative of *Burkholderia*-RFP infection) demonstrates that the majority of *B. hayleyella*
786 exposed hosts produce infected spores while a smaller fraction of *B. agricolaris* exposed
787 hosts produce infected spores. Co-infection by food bacteria is occasionally observed in
788 *B. agricolaris* infected spores (b). (*Klebsiella*-GFP shown in green, *Burkholderia*-RFP
789 shown in red, and calcofluor stain shown in grey). Top panels are image slices; bottom
790 panels are max intensity projections of z stacks. Scale bar: 10um.

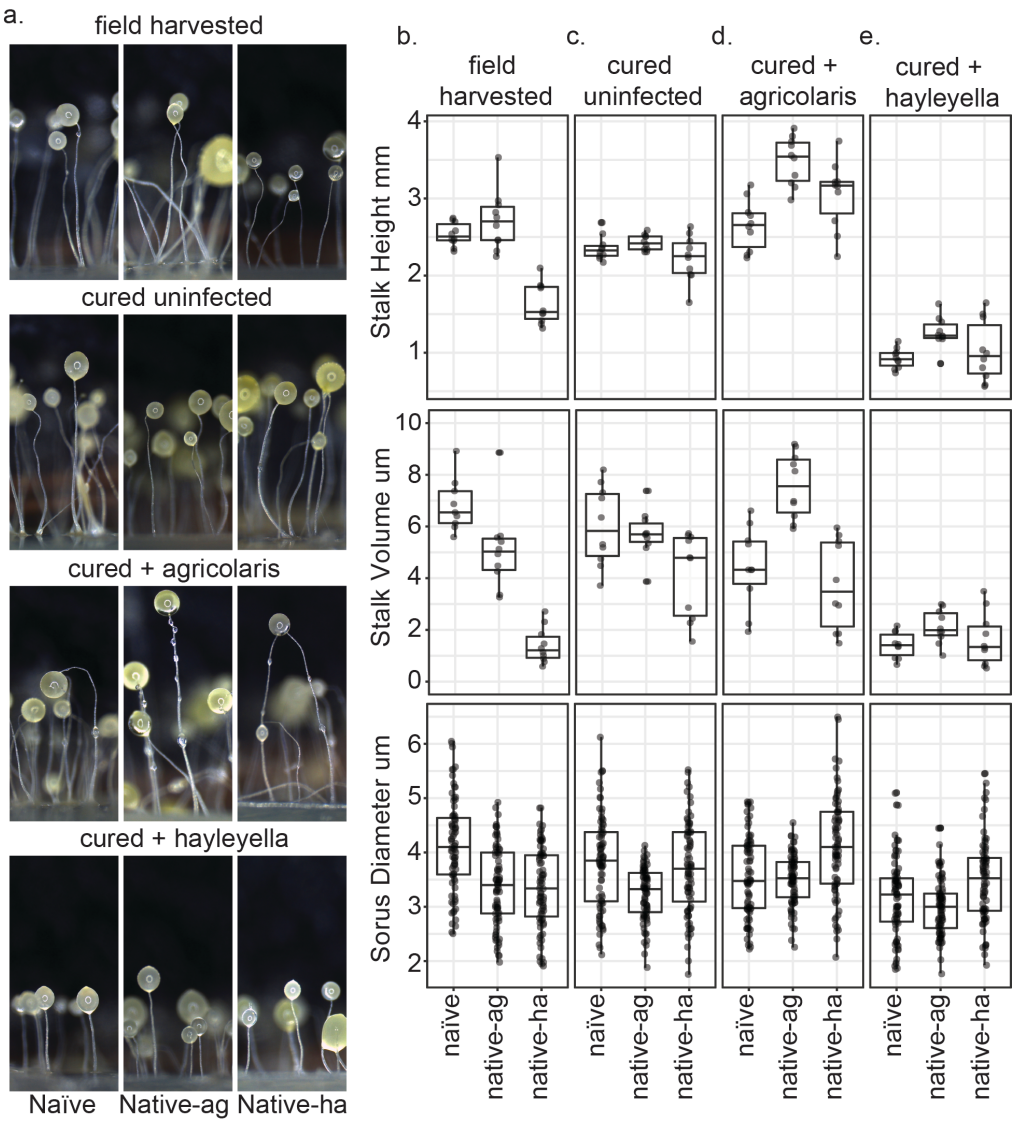


Figure 8. Fruiting body morphology is differentially altered by *Burkholderia* colonization. Macro photographs of fruiting bodies (a) show slightly different morphologies according to *Burkholderia* infection status. Sori measurements demonstrate that field collected native-*hayleyella* hosts produce shorter stalks and less voluminous sori (b). Cured hosts produce similar fruiting body measurements across host background (c). Cured hosts subsequently infected with *B. agricolaris* produce slightly taller stalks, which is most noticeable in cured and re-infected native-*agricolaris* hosts (d). Cured hosts subsequently infected with *B. hayleyella* all produce significantly shorter stalks with overall smaller fruiting body dimensions (e).

Supplemental Tables and Figures

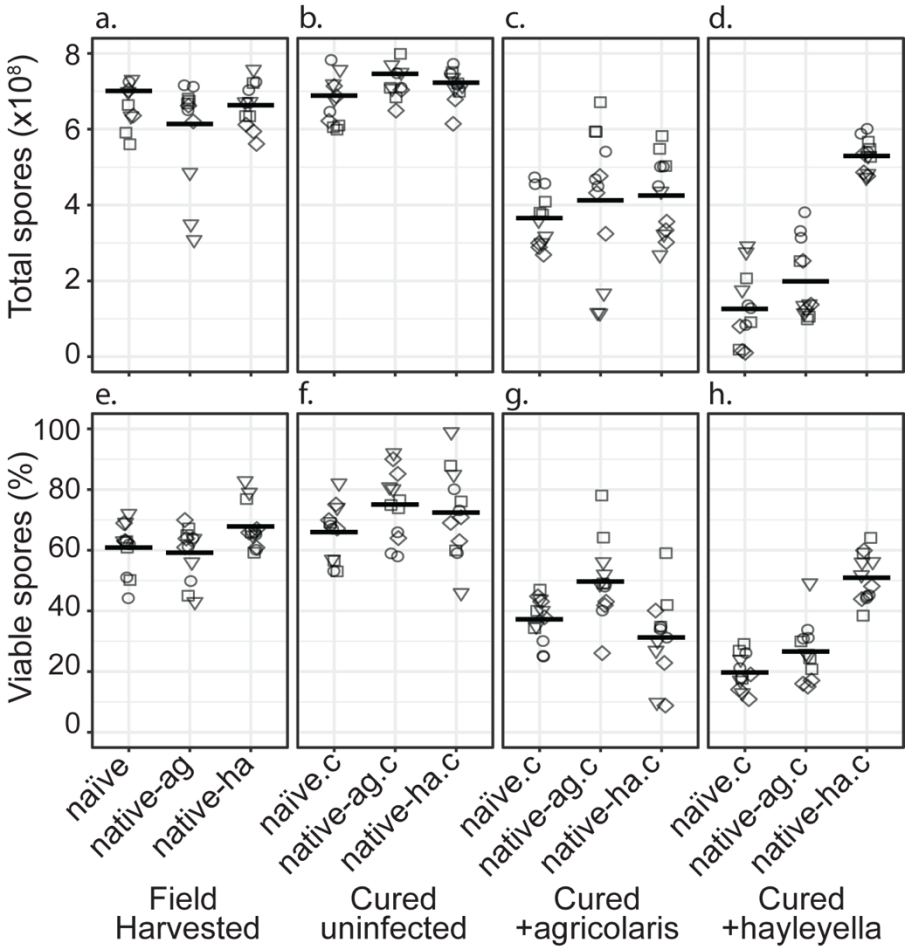


Figure S1. Total Spore Number and Percent of Viable Spores for *Burkholderia* Infections in Diverse Host Backgrounds. Total spores (top panel) and percent viable spores (bottom panel) were determined for naïve and native hosts in their field harvested (a), cured (b), *B. agricolaris* lab-infected (c), and *B. hayleyella* lab-infected state (d). Four clones were measured for each type with 3 replicates for each (squares, triangles, circles, and diamonds represent set 1-4 clones respectively). These data were used to determine total viable spores represented in Figure 2.

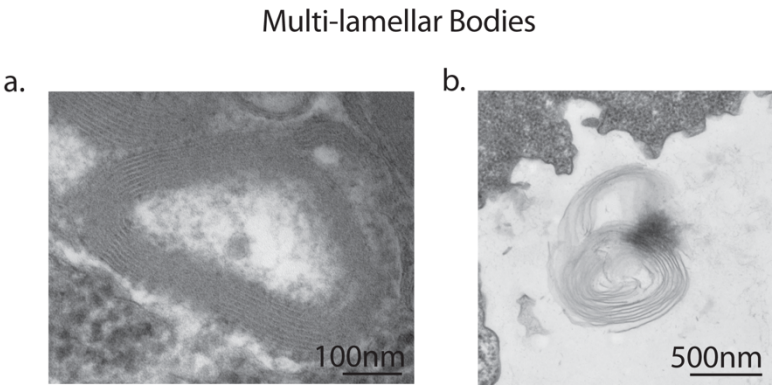


Figure S2. Multi-lamellar bodies excreted by vegetative amoebae. Transmission electron micrographs of vegetative amoebae identified multi-lamellar bodies inside uninfected amoebae, indicating successful digestion of bacterial food (a). Multi-lamellar bodies are eventually secreted into the surrounding medium (b).

		Fitness Measure		
	Question / Comparison	**Percent Viable Spores**	**Total Spore Production**	**Total Viable Spores****
	Are there fitness differences between field-collected states before curing?	**0.024**	0.11	0.062
a	**Naïve** vs. Native-***ag*** .	0.60	-	-
	Naïve vs. Native-***ha**** .	**0.035**	-	-
	Native-***ag*** . vs. Native-***ha****	**0.010**	-	-
	Are there fitness differences between field-collected states after curing?	0.10	0.84	0.32
b	**Naïve**.Cured vs. Native-***ag*** .Cured	-	-	-
	Naïve.Cured vs. Native-***ha*** . Cured	-	-	-
	Native-***ag*** . Cured vs. Native-***ha*** .Cured	-	-	-
	Is there an overall effect on native clones of curing with antibiotics?	**< 0.001**	**< 0.001**	**0.008**
c	Naïve.**Cured** vs. Naïve	0.76	0.99	0.95
	Native-*ag* .**Cured*** vs. Native-*ag.*	**0.0012**	**0.0027**	**< 0.001**
	Native-*ha* .**Cured** vs. Native-*ha.*	0.83	0.49	0.34
d	Does curing with antibiotics differentially affect the fitness of natural clones in terms of their *Burkholderia* infection status? (*i.e.,* Is there an interaction between *Burkholderia* infection in the field and curing with antibiotics?	**< 0.001**	**< 0.001**	**< 0.001**

* When significantly different, the red item with asterisk had the higher value

** Total Viable Spores are the product of Percent Viable Spores and Total Spore Production

N.B., Dashes (-) indicate that multiple comparisons were not performed because the main effect was not significant.

Table S1. Statistical results of three fitness measures assayed for field-collected amoeba clones and after curing with antibiotics. The three fitness measures were percent of spores that were viable, the total number of spores produced by a clone, and total viable spores. Total viable spores is the product of the other two measures. For each pairwise contrast, the essential difference in treatments is in boldface, and a treatment that is significantly higher than the other is marked with an asterisk and printed in red. Each of the fitness measures was analyzed with a set of Generalized Linear Mixed Models (GLMMs). This table gives the *p*-values for each question asked about main or interaction effects and the *post hoc* pairwise comparisons made, as relevant. Details about the statistical tests used can be found in the main text.

	Question / Comparison	Fitness Measure: Percent Viable Spores	Total Spore Production	Total Viable Spores*
a	Overall, are there fitness differences when either *Burkholderia* are added to cured clones?	**< 0.001**	**< 0.001**	**< 0.001**
b	Overall, are there fitness differences between cured clones when *B. agricolaris* is added*?	**< 0.001**	**< 0.001**	**< 0.001**
	Naïve.Cured+***agricolaris*** vs. Naïve.Cured*	**< 0.001**	**< 0.001**	**< 0.001**
	Native-*ag*.Cured+***agricolaris*** *vs.* Native-*ag*. Cured*	**< 0.001**	**< 0.001**	**< 0.001**
	Native-*ha*.Cured+***agricolaris*** vs. Native-*ha*.Cured*	**< 0.001**	**< 0.001**	**< 0.001**
c	Overall, are there fitness differences between cured clones when *B. hayleyella* is added*?	**< 0.001**	**< 0.001**	**< 0.001**
	Naïve.Cured+***hayleyella*** vs. Naïve.Cured*	**< 0.001**	**< 0.001**	**< 0.001**
	Native-*ag*.Cured+***hayleyella*** vs. Native-ag.Cured*	**< 0.001**	**< 0.001**	**< 0.001**
	Native-*ha*.Cured+***hayleyella*** vs. Native-ha.Cured*	**0.0001**	**< 0.001**	**< 0.001**
d	Is there an overall difference in fitness after the addition of *B. agricolaris* *vs. *B. hayleyella*?	**< 0.001**	**< 0.001**	**< 0.001**
	Naïve.Cured+***agricolaris**** vs. Naïve.Cured+***hayleyella***	**0.002**	**< 0.001**	**0.027**
	Native-*ag*.Cured+***agricolaris**** vs. Native-*ag*.Cured+***hayleyella***	**< 0.001**	**< 0.001**	**0.0002**
	Native-*ha*.Cured+***agricolaris*** vs. Native-*ha*.Cured+***hayleyella****	**0.0003**	0.12	**0.0037**
e	Is there an overall interaction between which *Burkholderia* is added and the native infection status of the amoeba clones?	**< 0.001**	**< 0.001**	**< 0.001**
	Do clones have higher fitness when their own *Burkholderia* are added?			
	Native-***ag***.Cured+*agricolaris* vs. N**aïve**.Cured+*agricolaris*	0.83	0.93	0.28
	Native-***ag***. Cured+*agricolaris* vs. Native-***ha***.Cured+*agricolaris*	0.43	1.0	0.45
	Native-***ha***.Cured+*hayleyella** vs. N**aïve**.Cured+*hayleyella*	**0.0009**	**< 0.001**	**< 0.001**
	Native-***ha***. Cured+*hayleyella** vs. Native-***ag***.Cured+*hayleyella*	*0.084*	**< 0.001**	**< 0.001**

* When significantly different, the red item with asterisk had the higher value

826 **Total Viable Spores is the product of Percent Viable Spores and Total Spore Production ⬆ ⬇

827 **Table S2. Statistical results of three fitness measures assayed for antibiotic-cured**
828 **amoeba clones after experimental addition of *Burkholderia*.** The three fitness measures
829 were again percent of spores that were viable, the total number of spores produced by a clone,
830 and total viable spores. Total viable spores is the product of the other two measures. For each
831 pairwise contrast, the essential difference in treatments is in boldface, and a treatment that is
832 significantly higher than the other is marked with an asterisk and printed in red. Each of the
833 fitness measures was analyzed with a set of Generalized Linear Mixed Models (GLMMs). This
834 table gives the *p*-values for each question asked about main or interaction effects and the *post*
835 *hoc* pairwise comparisons made, as relevant. Details about the statistical tests used can be
836 found in the main text.

	Question / Comparison	Stalk Length	Stalk Volume
	Overall, are there morphological differences between the three amoeba hosts?	**< 0.001**	**< 0.001**
a	Naïve* vs. Native-***ag***.	0.34	**0.0035**
	Naïve* vs. Native-***ha***.	**< 0.001**	**< 0.001**
	Native-***ag****. vs. Native-***ha***.	**< 0.001**	**< 0.001**
	Does curing with antibiotics affect the stalk morphology of the three amoeba hosts?	0.61	0.077
b	Naïve vs. Naïve.**Cured**.QS9	-	-
	Native-*ag*. vs. Native-*ag*.**Cured**.QS70	-	-
	Native-*ha* vs. Native-*ha*.**Cured**.QS11	-	-
	Is there an overall effect on stalk morphology when *B. agricolaris* is added to cured amoeba hosts?	**< 0.001**	0.95
c	Naïve.Cured vs. Naïve.Cured+***agricolaris***	0.34	-
	Native-*ag*.Cured vs. Native-*ag*.Cured+***agricolaris****	**< 0.001**	-
	Native-*ha*.Cured vs. Native-*ha*.Cured+***agricolaris****	**< 0.001**	-
	Is there an overall effect on stalk morphology when *B. hayleyella* is added to cured* amoeba hosts?	**< 0.001**	**< 0.001**
d	Naïve.Cured* vs. Naïve.Cured+***hayleyella***	**< 0.001**	**< 0.001**
	Native-*ag*.Cured* vs. Native-*ag*.Cured+***hayleyella***	**< 0.001**	**< 0.001**
	Native-*ha*.Cured* vs. Native-*ha*.Cured+***hayleyella***	**< 0.001**	**0.0012**
	Overall, is there an interaction between the amoeba hosts' native infection statuses and the species of *Burkholderia* added?	**0.001**	**< 0.001**
	Do amoeba hosts have a distinct stalk morphology when their own *Burkholderia* are added?		
e	Native-*ha*.Cured+**agricolaris*** vs. Native-*ha*.Cured+***hayleyella***	**< 0.001**	**0.01**
	Native-*ag*.Cured+***agricolaris**** vs. Native-ag.Cured+***hayleyella***	**< 0.001**	**< 0.001**
	Does the Native-naïve clone have a distinct stalk morphology when *Burkholderia* are added?		
	Native-naïve. Cured+***agricolaris**** vs. Native- naïve.Cured+***hayleyella***	**< 0.001**	**< 0.001**

* When significantly different, the red item with asterisk had the higher value

* *Total Viable Spores is the product of Percent Viable Spores and Total Spore Production

N.B., Dashes (-) indicate that multiple comparisons were not performed because the main effect was not significant.

Table S3. Statistical results for stalk morphology. Each of the stalk measures was analyzed with a set of Generalized Linear Mixed Models (GLMMs). This table gives the *p*-values for each question asked about main or interaction effects and the *post hoc* pairwise comparisons made, as relevant. For each pairwise contrast, the essential difference in treatments is in boldface, and a treatment that is significantly higher than the other is marked with an asterisk and printed in red. Details about the statistical tests used can be found in the main text. One clone of each native type was tested: QS9 naïve; QS70 *ag*-infected; QS11 *ha*-infected.

	Question / Comparison	Sorus Diameter	Sorus Volume
	Overall, are there morphological differences between the three amoeba hosts?	**< 0.001**	**< 0.001**
a	Naïve* vs. Native-***ag***.	**< 0.001**	**< 0.001**
	Naïve* vs. Native-***ha***.	**< 0.001**	**< 0.001**
	Native-***ag***. vs. Native-***ha***.	0.98	0.99
	Does curing with antibiotics affect the sorus morphology of the three amoeba hosts?	0.63	0.80
b	Naïve vs. Naïve.**Cured**	-	-
	Native-*ag* vs. Native-*ag*.**Cured**	-	-
	Native-*ha* vs. Native-*ha*.**Cured**	-	-
	Is there an overall effect on sorus morphology when *B. agricolaris* is added to cured amoeba hosts?	**< 0.001**	**< 0.001**
c	Naïve.Cured vs. Naïve.Cured+***agricolaris***	0.30	0.45
	Native-*ag*.Cured vs. Native-*ag*.Cured+***agricolaris***	0.31	0.25
	Native-*ha*.Cured vs. Native-*ha*.Cured+***agricolaris****	**0.012**	**0.036**
	Is there an overall effect on sorus morphology when *B. hayleyella* is added to cured amoeba hosts?	**< 0.001**	**< 0.001**
d	Naïve.Cured* vs. Naïve.Cured+***hayleyella***	**< 0.001**	**< 0.001**
	Native-*ag*.Cured vs. Native-*ag*.Cured+***hayleyella***	0.53	0.34
	Native-*ha*.Cured vs. Native-*ha*.Cured+***hayleyella***	0.52	0.63
	Overall, is there an interaction between the amoeba hosts' native infection status and the species of *Burkholderia* added?	**0.0004**	**0.0014**
	Do amoeba have a distinct sorus morphology when their own *Burkholderia* are added?		
e	Native-*ha*.Cured+**agricolaris*** vs. Native-*ha*.Cured+***hayleyella***	**< 0.001**	**< 0.001**
	Native-*ha*.Cured+**agricolaris*** vs. Native-*ha*.Cured+***hayleyella***	**0.0005**	**< 0.001**
	Does the naïve clone have a distinct sorus morphology when *Burkholderia* are added?		
	Native-naïve.Cured+***agricolaris**** vs. Native-naïve.Cured+***hayleyella***	**0.018**	**0.011**

* When significantly different, the red item with asterisk had the higher value

* *Total Viable Spores is the product of Percent Viable Spores and Total Spore Production

N.B., Dashes (-) indicate that multiple comparisons were not performed because the main effect was not significant.

Table S4. Statistical results for sorus morphology. Each of the spore measures was analyzed with a set of Generalized Linear Mixed Models (GLMMs). This table gives the *p*-values for each question asked about main or interaction effects and the *post hoc* pairwise comparisons made, as relevant. For each pairwise contrast, the essential difference in treatments is in boldface, and a treatment that is significantly higher than the other is marked with an asterisk and printed in red. Details about the statistical tests used can be found in the main text. One clone of each native type was tested: QS9 naïve; QS70 *ag*-infected; QS11 *ha*-infected.