Burkholderia bacteria infectiously induce the proto-farming symbiosis of *Dictyostelium* amoebae and food bacteria

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Symbiotic associations can allow an organism to acquire novel traits by accessing the genetic repertoire of its partner. In the *Dictyostelium discoideum* farming symbiosis, certain amoebas (termed "farmers") stably associate with bacterial partners. Farmers can suffer a reproductive cost but also gain beneficial capabilities, such as carriage of bacterial food (proto-farming) and defense against competitors. Farming status previously has been attributed to amoeba genotype, but the role of bacterial partners in its induction has not been examined. Here, we explore the role of bacterial associates in the initiation, maintenance, and phenotypic effects of the farming symbiosis. We demonstrate that two clades of farmer-associated *Burkholderia* isolates colonize *D. discoideum* nonfarmers and infectiously endow them with farmer-like characteristics, indicating that *Burkholderia* symbionts are a major driver of the farming phenomenon. Under food-rich conditions, *Burkholderia*-colonized amoebas produce fewer spores than uncolonized counterparts, with the severity of this reduction being dependent on the *Burkholderia* colonizer. However, the induction of food carriage by *Burkholderia* colonization may be considered a conditionally adaptive trait because it can confer an advantage to the amoeba host when grown in food-limiting conditions. We observed *Burkholderia* inside and outside colonized *D. discoideum* spores after fruiting body formation; this observation, together with the ability of *Burkholderia* to colonize new amoebas, suggests a mixed mode of symbiont transmission. These results change our understanding of the *D. discoideum* farming symbiosis by establishing that the bacterial partner, *Burkholderia*, is an important causative agent of the farming phenomenon.

Burkholderia | *Dictyostelium* | symbiosis | mutualism | social amoeba

Symbiotic interactions are ubiquitous in nature and can play a central role in the evolutionary trajectory of organisms. For instance, symbiosis can drive rapid lateral procurement of novel traits as interacting organisms gain access to the genetic capabilities of their partner (1, 2). The evolutionary power of symbiosis is apparent in the many major life forms that owe their very existence to past and present symbiotic partnerships (3, 4). A famous example is the emergence of eukaryotes through their ancestor's acquisition of bacteria that subsequently evolved into organelles indispensable for energy generation (5). Although many classic examples of symbiosis are conspicuously mutualistic, the characteristics of other symbiotic associations can be complex, dynamic, and less definable. In some cases, symbionts have good or bad effects on their host that vary depending on genotypic and environmental details (6, 7). The length and transmission mode of symbiosis also can have strong effects on the selection and evolution of partner traits. Although older symbiotic associations are often obligate and stable, recent associations can be transient and protean (8). Additionally, vertical transmission may favor mutualistic interactions, whereas horizontal transmission can allow the emergence and spread of more pathogenic characteristics (9–11). However, as a whole, the fates of symbioses are often the result of a delicate balance between mutualism and pathogenesis, requiring pathogenic characteristics at the least to facilitate infection and beneficial properties to promote maintenance (12, 13). Indeed, many cases of mutualistic associations are thought to have evolved from ancient parasitic infections (14, 15). Examining nascent, malleable, or less definable forms of symbiosis may provide insight into the mechanisms that promote or corrode this balance and their subsequent evolutionary consequences.

Amoeba–bacteria interactions make a promising system for gaining insight into diverse and dynamic symbiotic relationships. Amoebas interact with bacteria in multiple ways. Most apparently, they are predators of bacteria. However, other amoeba–bacteria interactions are less favorable for the amoebas. Some bacteria can evade amoeba phagocytosis and thereby diminish amoeba predatory prowess and food acquisition (16). Still worse, amoebas can fall victim to bacterial processes or exploitation, with some bacteria producing products detrimental to amoeba fitness or surviving phagocytosis to invade and multiply within amoeba cells (17, 18). There also are stable symbiotic interactions between amoebas and bacteria in which the origins, mechanisms,

Significance

Symbionts can provide hosts with many advantages including defensive capabilities and novel nutrients. However, symbionts may begin as pathogens that only subsequently become beneficial. In the *Dictyostelium discoideum* farming symbiosis some amoebas stably associate with bacterial partners. We demonstrate that amoeba-associated *Burkholderia* can initiate a farming symbiosis with naive amoeba hosts. *Burkholderia* decreases amoeba spore productivity in food-rich conditions but, because of the induction of bacterial food carriage, sometimes increases spore productivity in food-scarce conditions. Detrimental effects of *Burkholderia* colonization differ among *Burkholderia* genotypes and, in some cases, between new and old amoeba hosts, suggesting some coevolution within the association. These results suggest that *Burkholderia* exerts both pathogenic and mutualistic effects on its host in conditionally dependent ways.

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and impacts on both species are less defined. For instance, several bacterial endosymbionts inhabit amoebas, incurring variable and not always obvious consequences to the amoeba host (19–22). In addition, certain isolates of the soil-dwelling amoeba *Dictyostelium discoideum* persistently associate with bacteria, an association that has beneficial or detrimental outcomes depending on the environmental conditions (23–25). Amoebas can serve as environmental reservoirs for bacterial pathogens (26, 27) or as training facilities for the adaptation of bacteria to evade eukaryotic phagocytosis or to survive intracellularly after phagocytosis (17, 28). Although, for the eukaryotic host, some of these interactions may have decidedly unfortunate outcomes, such as the emergence of bacterial pathogenesis through intracellular adaptation, others may illuminate important evolutionary advances, such as the transition of bacterial endosymbionts into organelles that provide novel functionality. Thus, the diversity and persistence of amoeba–bacteria interactions may have several ecological and health-related consequences.

The stable association between bacteria and some wild isolates of *D. discoideum* supplies a particularly interesting study system for elucidating symbiotic causes and consequences. *D. discoideum* is a soil-dwelling amoeba that transitions between solitary and social life stages (29). In the solitary stage, *D. discoideum* cells consume bacteria through phagocytosis and divide by binary fission. When food is exhausted, amoebas coaggregate, ultimately forming a multicellular structure called a "fruiting body." As the fruiting body develops, ∼20% of the cells sacrifice themselves to form an erect stalk. The remaining cells ascend the stalk, forming a globular sorus at the top where they differentiate into sturdy spores (29). In the sorus, spores are positioned for dispersal into more favorable environments, presumably through contact and transport upon animals (30). Multiple wild isolates of the amoeba *D. discoideum*, but not all of them, have been found to be stably associated with several bacteria species (24). These amoebas persistently carry both edible and inedible bacteria with them through the social stage and are termed "farmers" because of their ability to reseed new environments with a food source (23, 24). Interestingly, farmer isolates produce fewer spores under optimal conditions than their non–bacteria-carrying counterparts; however, this cost is countered by farmers' advantage in being able to replenish their food supply when dispersing to food-poor environments (24). Additionally, some of the bacterial isolates that farmers carry produce compounds detrimental to nonfarmer clones but not to their host farmer, giving the host farmer a competitive advantage in a mixed population (23, 25).

The ease of *D. discoideum* manipulation coupled with the variability inherent in the farming symbiosis provide a promising platform for addressing questions concerning symbiosis between microbes and eukaryotes. For instance, are mutualistic associations and their resulting phenotypes driven by the host, by the bacteria, or by a very specific interaction between the two? How much coevolution is required to reach a beneficial outcome? In aphids and other insects, defensive symbionts can sweep through populations and be horizontally transferred to new hosts (31, 32). Even *Buchnera*, a vertically transferred obligate symbiont of aphids, still confers its benefits to new hosts after experimental infection (33). Taken together, this horizontal transfer suggests that little coevolution may be needed for the formation of novel symbiosis. However, in some plant–rhizobia or –mycorrhizal associations, the symbiont phenotype can vary substantially among hosts, and the outcome of the association is determined by the interaction of host and symbiont-derived factors (34–38). Similarly, a synergistic interplay between host and symbiont components mediates the initiation and persistence of the bobtail squid–*Vibrio fischeri* symbiosis (39–41). For the *D. discoideum* farming symbiosis, it previously had been assumed that farmers were genetically distinct from nonfarmers, suggesting the role of a host-specific factor in establishing the symbiosis (24). This study aims to characterize more thoroughly the partner dynamics within the *D. discoideum* farming symbiosis by specifically analyzing the role of bacterial associates in farming phenotypes.

To determine the role bacterial partners play in farming, we first examined the diversity of bacterial passengers associated with our present collection of stable farmer clones. We confirmed that several different bacterial species can be isolated from farmer *D. discoideum*; however isolates belonging to the *Burkholderia* genus were ubiquitous among our tested farmers. These *Burkholderia* isolates fail to support amoeba growth when provided as the only food source and therefore are considered inedible. Because of their prevalence in our farmer clones and because *Burkholderia* species form symbiotic relationships with diverse organisms, we hypothesized that these *Burkholderia* isolates could be crucial for the symbiotic relationship with *D. discoideum* that results in the farming phenomenon (42). Therefore we asked whether nonfarmer *D. discoideum* could be colonized by farmer-associated *Burkholderia* isolates and whether colonization could induce secondary bacterial carriage. We established that the *Burkholderia* associated with *D. discoideum* fall into two distinct phylogenetic clades. We found that the tested *Burkholderia* isolates from each clade robustly colonized nonfarmer *D. discoideum* sori, with this colonization persisting through multiple rounds of *D. discoideum* spore dispersal, germination, and vegetative growth. Like their farmer counterparts, nonfarmers colonized with *Burkholderia* can carry bacterial food, allowing them to reseed new territories with food bacteria following spore dispersal. Inversely, removal of *Burkholderia* from wild farmers by antibiotic treatment results in the loss of detectable bacterial food carrying. These findings suggest that *Burkholderia* colonization drives secondary bacterial carriage (farming) in *D. discoideum*. In total, these results suggest that specific *Burkholderia* isolates stably colonize *D. discoideum* and induce a novel adaptive trait of ecological relevance, the carriage of bacterial food. Our initial evidence suggests that the consequences of *Burkholderia* carriage may differ according to symbiont and host genotypes. For instance, some *Burkholderia* isolates impose a higher cost to their hosts, and the extent of this cost appears to be more severe for newer hosts than for the original host. We also observe *Burkholderia* (and occasionally, our laboratory food bacterium, *Klebsiella pneumoniae*) inside colonized *D. discoideum* spores after fruiting body formation. This observation, together with *Burkholderia*'s ability to be horizontally transmitted to new hosts and to associate stably with old hosts, suggests a mixed mode of *Burkholderia* transmission. The less severe fitness costs exerted by *Burkholderia* colonization in the original hosts suggests that long-term vertical transmission may lead to compensatory host adaptation.

Results

***Burkholderia* Species Are Associated with Stable *D. discoideum* Farmer Clones in Our Collection.** To identify the persistence and prevalence of bacterial species in our frozen stock collection of farmer clones, we reisolated bacterial colonies from farmer *D. discoideum* sorus contents and sequenced their 16S rRNA gene for putative identification. We found that some farmer lines grown from single spores no longer retained their bacterial carriage phenotype (two lines from 15 tested farmer stocks). Because these lines originated from a single spore from a farmer population, this observation may suggest instability or incomplete penetrance of the trait among individuals of the same genotype and even from the same fruiting body. Clones positive for bacterial carriage were found to carry several bacterial types including various species of *Burkholderia*, *Stenotrophomonas*, and *Flavobacterium*, consistent with previous observations (Table S1) (23–25). Not surprisingly, given the well-documented trait of food carriage in farmer clones, we also frequently isolated *K. pneumoniae*, our bacterial food source for *Dictyostelium*,

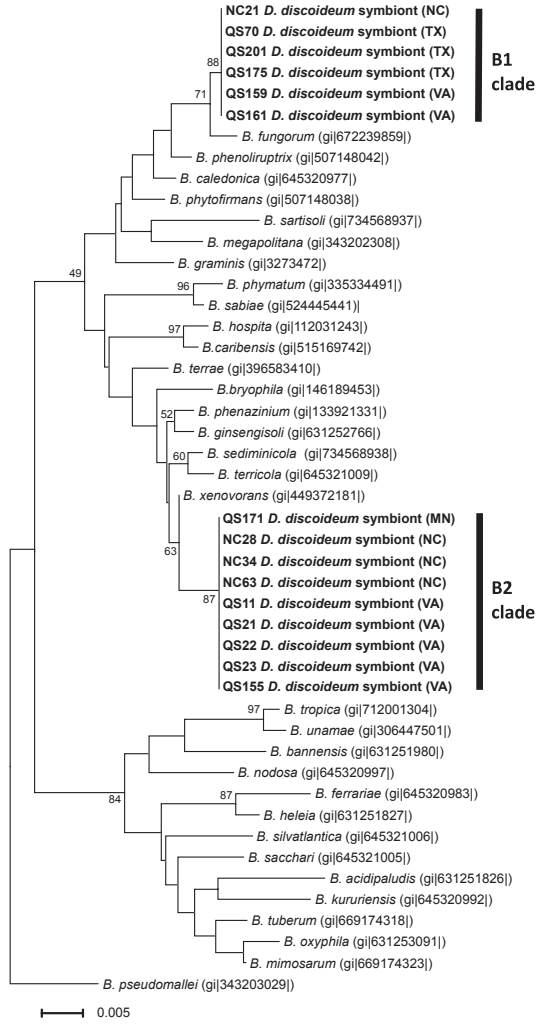

Fig. 1. Burkholderia phylogeny. Phylogenetic tree based on 400-nt 16S rRNA gene sequences showing relatedness of *D. discoideum*-associated *Burkholderia* isolates with environmental *Burkholderia* species using the pathogenic *Burkholderia pseudomallei* as an outgroup. The current collection of *D. discoideum*-associated *Burkholderia* isolates falls within two distinct clades, referred to here as clades B1 and B2.

from farmer sorus contents (24). One previously unappreciated aspect of farmer-carried bacteria that emerged from this round of reisolation and sequencing was that isolates of *Burkholderia* were present in every stable farmer we looked at from the current collection (Table S1). Although this prevalence suggests a key role for *Burkholderia*, our sampling was not exhaustive and therefore does not rule out the existence of farmer clones without *Burkholderia*.

Two Independent Clades of *Burkholderia* Have Colonized *D. discoideum*. To examine the diversity of *Burkholderia* isolates associated with farmer *D. discoideum* and their relationship with other *Burkholderia* species, we constructed a 16S rRNA gene phylogeny (Fig. 1). Among our samples of farmer-borne *Burkholderia* isolates, two distinct clades emerged, B1 and B2, suggesting that there are at least two independent origins of the *Burkholderia* symbiosis with *D. discoideum*. Both clades show close relationships with species belonging to the plant-beneficial-environmental *Burkholderia* cluster (42). These *Burkholderia* are widely distributed geographically: Individuals from both clades have been sampled thus far in North Carolina and Virginia, with additional B1 isolates from Texas and B2 isolates from Minnesota (Fig. 1). For the purpose of this article, we identify our bacterial isolates with the clade within which they are clustered and by the *D. discoideum* clone from which they were isolated (e.g., *Burkholderia* from clade B1 isolated from QS70 is designated "B1qs70").

***Burkholderia* Isolates Associated with Wild *D. discoideum* Farmers Confer Bacterial Carriage to Noncarriers.** In light of the prevalence of *Burkholderia* species in farmer clones of *D. discoideum*, we hypothesized that *Burkholderia* itself, rather than a distinct *D. discoideum* genotype, is the predominant initiator or driver of the farmer phenomenon. To test this prediction, we exposed 10 wild farmers and 10 nonfarmers to a collection of six farmer-associated *Burkholderia* isolates (three isolates per clade) and to a control collection of bacterial strains not typically found to be associated with farmers (Fig. 2*A*). After the *D. discoideum* clones completed their social cycle under the given exposure conditions, we tested for the presence of bacteria in their sorus contents by plating individual sori onto nutrient medium and observing bacterial growth after incubation (see representative images in Fig. 2*B*). We found that exposure to farmer-associated *Burkholderia* isolates, but not to unassociated bacterial strains, resulted in the presence of bacteria in nonfarmer *D. discoideum* fruiting body heads (sori) after development (Fig. 2*A*). In particular,

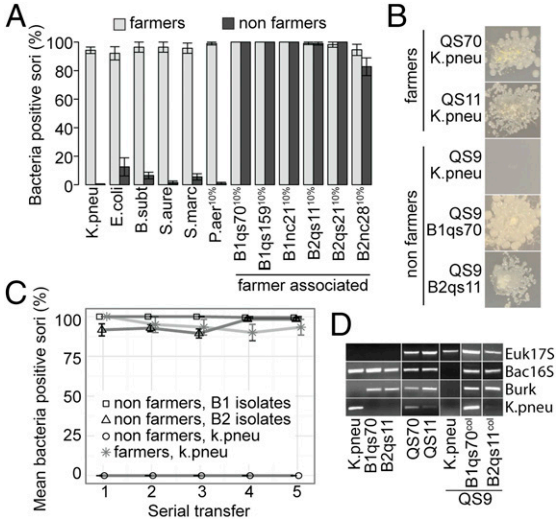

Fig. 2. Exposure of *D. discoideum* to *Burkholderia* results in stable bacterial carriage. (*A*) The percent of bacteria-positive sori from 10 farmers and 10 nonfarmers after exposure to *K. pneumoniae*, *E. coli*, *Bacillus subtilis*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and the indicated *Burkholderia* isolates was detected by spotting individual sorus contents on nutrient medium and observing bacterial growth. All the tested *Burkholderia* isolates and the *P. aeruginosa* strain do not support *D. discoideum* growth and development without the addition of a bacterial food source. Therefore, to ensure *D. discoideum* growth during exposure to these strains, they were mixed at 10% by volume with a culture of *K. pneumoniae* after both cultures were preset to an OD_{600} of 2 in KK2 buffer (denoted by 10%). The other four bacteria were edible and did not need to be mixed with *K. pneumonia* for *D. discoideum* to proliferate. Error bars represent SEM. (*B*) Images of bacterial growth after the contents of one sorus from the indicated *D. discoideum* clone were plated on nutrient medium. (*C*) Percent of bacteria-positive sori from six farmer and six nonfarmer *D. discoideum* clones after exposure to the indicated bacterial isolates (transfer 1) and after subsequent social cycles on *K. pneumoniae* alone (transfers 2–5). Error bars represent SEM. (*D*) Representative agarose gel images of PCR amplification of genes specific to eukaryotes, eubacteria, *Burkholderia*, and *Klebsiella* from DNA isolated from the sorus contents of farmer (QS70 and QS11) and nonfarmer (QS9) *D. discoideum* clones (the superscript "col" indicates precolonization with the indicated *Burkholderia* isolate).

our food bacterium *K. pneumoniae* does not induce its own carriage. This result demonstrates that farmer-borne *Burkholderia* can induce *D. discoideum* to carry bacteria through the social stage.

We next examined the stability of this trait through multiple social cycles under standard laboratory conditions. To do so, we harvested spores produced after exposure of six nonfarmers and six farmers to *Burkholderia* isolates or *K. pneumoniae* and passaged them onto *K. pneumoniae* alone for four additional social cycles (spores produced after each transfer were harvested for the subsequent transfer). We found that nonfarmers exposed once to *Burkholderia* retained their ability to carry bacteria through multiple social cycles, akin to the stability of bacterial carriage found in wild farmer clones (Fig. 2*C*).

We also tested whether representative non-*Burkholderia* bacterial species associated with farmer clones (*Stenotrophomonas* and *Flavobacterium* strains isolated from this study and two *Pseudomonas fluorescens* strains isolated previously) could confer bacterial carriage to nonfarmers (24). We found that these farmer-associated non-*Burkholderia* isolates could induce moderate levels of bacterial carriage in nonfarmers (Fig. S1). This moderate level of bacterial carriage persisted through multiple social cycles for *Flavobacterium*-exposed nonfarmers but did not persist for *Stenotrophomonas*- or *Pseudomonas*-exposed nonfarmers (Fig. S1). These results suggest that, in addition to *Burkholderia*, other farmer-associated bacteria may have the ability to induce bacterial carriage to differing degrees of strength and stability.

***Burkholderia* Colonization Induces Secondary Bacterial Carriage.** To confirm *Burkholderia* colonization of nonfarmers, we isolated and identified bacteria from the sorus contents of a nonfarmer clone independently exposed to B1qs70, B1nc21, B2qs11, or B2nc28. In each case, we were able to confirm the presence of the original *Burkholderia* isolate by 16S rRNA gene sequencing. Henceforth, we refer to nonfarmer clones that have been exposed to and carry *Burkholderia* as "*Burkholderia*-colonized nonfarmers" to distinguish them from their nonexposed counterparts and from original wild farmers. We also isolated and identified our bacterial food source, *K. pneumoniae*, after *Burkholderia* exposure, suggesting that colonization of *D. discoideum* by *Burkholderia* induces carriage of bacterial food. Moreover, we can amplify *Burkholderia*, and in most cases *K. pneumoniae*, DNA directly from the sorus of wild farmers and *Burkholderia*-exposed nonfarmers using genus-specific PCR primers on total sorus content DNA extractions (Fig. 2*D* and Table S2). Carriage of additional bacterial species is not limited to *K. pneumoniae*, because sorus contents from farmers and *Burkholderia*-exposed nonfarmers contain red-pigmented bacteria after growth on a red strain of *Serratia marcescens* (Fig. S2). *S. marcescens* also is unable to induce carriage on its own (Fig. 2*B*). These results indicate that *Burkholderia* stably colonizes *D. discoideum* and to some extent induces secondary bacterial carriage. The induction of secondary bacterial carrying by *Burkholderia* could explain the occasional identification of multiple bacterial species from wild farmers.

***Burkholderia* Colonization Imposes a Cost in Spore Productivity Under Food-Abundant Conditions.** Farmer clones produce fewer spores than nonfarmers under standard laboratory conditions (i.e., with the provision of abundant bacterial food) (24). Exposure to specific *Burkholderia* isolates and their byproducts also was shown to harm nonfarmers but not their host farmers (25). To determine the cost imposed by *Burkholderia* colonization on nonfarmers, we compared the spore production of four farmers, six uncolonized nonfarmers, and six *Burkholderia*-colonized nonfarmers (colonized via pregrowth on 10% B1qs70, B1nc21, B2qs11, or B2nc28 and 90% *K. pneumoniae*). Spores were plated on nutrient agar medium with live *K. pneumoniae* (normal culture conditions), which replicates rapidly to form a lawn on the plate and provides an abundance of bacterial food for the amoebas. After development on live *K. pneumoniae*, total *D. discoideum* spores were counted (Fig. 3*A*). In line with previous results, we found that uncolonized nonfarmers typically produce more spores than farmer clones [L-ratio (likelihood-ratio test) = 4.62, df = 1, $P = 0.03$] (24). However, *Burkholderia*-colonized nonfarmers are more like farmers, in that they produce fewer spores on average than their uncolonized counterparts (L-ratio = 88.9, df = 1, $P < 0.001$) (Fig. 3*A*). The severity of this reduction is dependent on the identity of the *Burkholderia* colonizer, with the two tested isolates of *Burkholderia* from clade B2 being more detrimental for spore production than the two tested isolates from clade B1 (L-ratio = 47.94, df = 1, $P < 0.001$) (Fig. 3*A*). However, the cost in spore-productivity associated with *Burkholderia* isolates from clade B2 can be alleviated somewhat by colonizing at a lower dosage (0.1% rather than 10%) (L-ratio = 103.09, df = 1, $P < 0.001$) (Fig. S3). Consistent with previous observations that host farmers are more resilient than nonfarmers to the detrimental effects of their *Burkholderia* colonizer (25), we found that the host farmers QS11 and NC28 fared better than nonfarmers when preexposed to 0.1% of their respective *Burkholderia* isolate (L-ratio = 4.47, df = 1, $P < 0.04$) (Fig. S3). Thus, the costs induced by *Burkholderia* colonization appear to depend on the *Burkholderia* and *D. discoideum* genotypes and on the dose of *Burkholderia* used to initiate colonization. Nonfarmers appear to be particularly harmed by *Burkholderia* from clade B2, suggesting that initiation of new clade B2 symbioses may be difficult. Hosts of clade B2 may have evolved to be less harmed by the interaction, or, alternatively, some host genotypes may display higher resiliency to the costs of the association at onset.

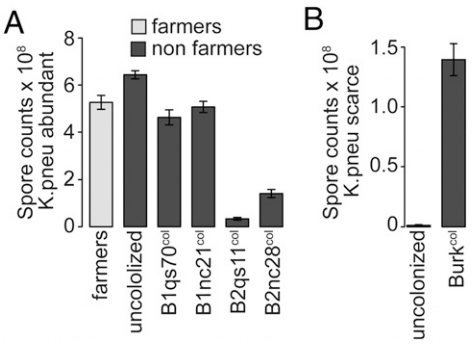

Fig. 3. Colonization of *D. discoideum* with *Burkholderia* confers differential costs and benefits depending on dispersal conditions. (*A*) Total *D. discoideum* spore counts after growth on live *K. pneumoniae* (K.pneu abundant) for three replicates each of four farmers, six uncolonized nonfarmers, and six nonfarmers colonized via pregrowth with 10% of the indicated *Burkholderia* isolate (^{col}). Error bars represent SEM. (*B*) Total spore counts after growth on dead *K. pneumoniae* (K.pneu scarce) for three replicates each of six uncolonized nonfarmers (uncolonized) and six nonfarmers colonized with *Burkholderia* via pregrowth with 10% B1qs70, B1nc21, B2qs11, or B2nc28 (Burk^{col}). Error bars represent SEM.

***Burkholderia* Provides a Benefit to Its Amoeba Host Under Food-Scarce Conditions.** Farmer amoebas have been shown previously to produce more spores than nonfarmers in food-scarce environments, a benefit attributed to their ability to carry food bacteria into their new environment after spore dispersal (24). Because *Burkholderia* colonization induces secondary bacterial carriage to nonfarmers, we expected colonized nonfarmers also to produce more spores than their uncolonized counterparts in food-poor environments. To quantify this potential advantage, we compared the number of spores produced by *Burkholderia*-colonized nonfarmers and by their uncolonized counterparts

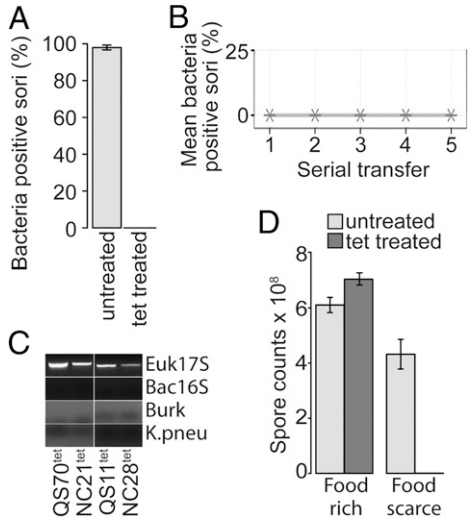

Fig. 4. *Burkholderia* and its associated effects can be eliminated from original hosts by antibiotic treatment. (*A*) Percent of bacteria-positive sori for 10 farmers with or without prior tetracycline treatment. Error bars represent SEM. (*B*) Percent of bacteria-positive sori over the course of five social cycles with *K. pneumoniae* for 10 farmers pretreated with tetracycline. Error bars represent SEM. (*C*) PCR amplification of eukaryote-, eubacteria-, *Burkholderia*-, and *Klebsiella*-specific genes from DNA isolated from the sorus contents of representative tetracycline-treated farmers. (*D*) Total spore counts after growth on live (food-rich) or dead (food-scarce) *K. pneumoniae* for three replicates of four farmers with or without prior tetracycline treatment. Error bars represent SEM.

after development on nutrient agar medium under food-scarce conditions. Food-scarce conditions were created by coculturing spores with a small amount of heat-killed *K. pneumoniae*, thereby limiting the amount of bacterial food provided. We find that under these conditions *Burkholderia* colonization significantly increases nonfarmer spore productivity (L-ratio = 140.4, df = 1, $P < 0.0001$) (Fig. 3*B*). Together with the results of the previous section, these results show that the costs and benefits of novel *Burkholderia* colonization are condition dependent, just as they are for farmers (24), suggesting that colonization could be antagonistic or mutualistic for *D. discoideum* depending on the given environmental conditions.

***Burkholderia* Colonization and Its Associated Effects Can Be Eliminated from Original Farmer Clones by Antibiotic Treatment.** We next wanted to test whether *Burkholderia* colonization and its associated costs and benefits could be eliminated from wild farmer clones. We found that all our tested farmer-associated *Burkholderia* isolates were sensitive to tetracycline. Thus, we cleared *Burkholderia* from farmer clones by growing them on nutrient agar medium containing tetracycline. By passaging a small number of farmer spores for two rounds through the social stage on tetracycline plates, followed by transfer to non–antibiotic-containing plates and testing for bacterial carriage, we found that we were no longer able to detect bacterial growth from their sorus contents (Fig. 4*A*). Our inability to detect bacterial growth from tetracycline-treated farmer sori was consistent over multiple social cycles during serial transfer (in the absence of continued tetracycline treatment) (Fig. 4*B*). Furthermore, we no longer could amplify *Burkholderia*- or *Klebsiella*-specific DNA from tetracycline-treated farmer sori (see Fig. 4*C* for representative images). This finding suggests that the elimination of *Burkholderia* by antibiotic treatment may permanently remove the ability of farmers to carry *K. pneumoniae* through their social stage. Additionally, compared with their nontreated counterparts, tetracycline-treated farmers produced significantly more spores in food-abundant conditions (L-ratio = 5.08, df = 1, $P = 0.024$) and fewer spores in food scarce conditions (L-ratio = 53.7, df = 1, $P \leq 0.001$) (Fig. 4*D*). In sum, they behaved exactly like nonfarmers in their inability to transport bacteria and in their spore fitness. Together, these data support the role of *Burkholderia* in traits associated with the farming phenomenon.

***Burkholderia* Localization Is Similar in Nonfarmer and Host Farmer Spores.** To visualize the association between *Burkholderia* and *D. discoideum*, we colonized a nonfarmer (QS9) and the original host farmer after tetracycline treatment (QS70-tet) by plating spores in a mixture of B1qs70-RFP and *K. pneumoniae*-GFP. After fruiting body development, we imaged colonized and uncolonized (grown with *K. pneumoniae*-GFP only) spores stained with calcofluor using confocal microscopy. *Burkholderia* and, less frequently, *K. pneumoniae* could be observed inside colonized host farmer and nonfarmer spores (see representative images in Fig. 5). These results show, for the first time to our knowledge, that *Burkholderia* can be carried intracellularly by *D. discoideum*. Interestingly, not all spores appear to be colonized by *Burkholderia*, and those that do often contain multiple bacterial cells, suggesting that *Burkholderia* may continue to replicate within amoeba cells. Additionally, we can observe fluorescent bacteria floating in the extracellular fluid of colonized sorus contents, suggesting that bacteria also may associate with amoebas externally or are consistently liberated from amoeba cell contents.

Discussion

This study demonstrates the ability of *Burkholderia* of two different clades to induce the farming symbiosis of *D. discoideum*. First, we showed that nonfarmers can be transformed into a farmer-like state by exposure to farmer-borne *Burkholderia* isolates. Like farmers, nonfarmers exposed to *Burkholderia* (*i*) stably

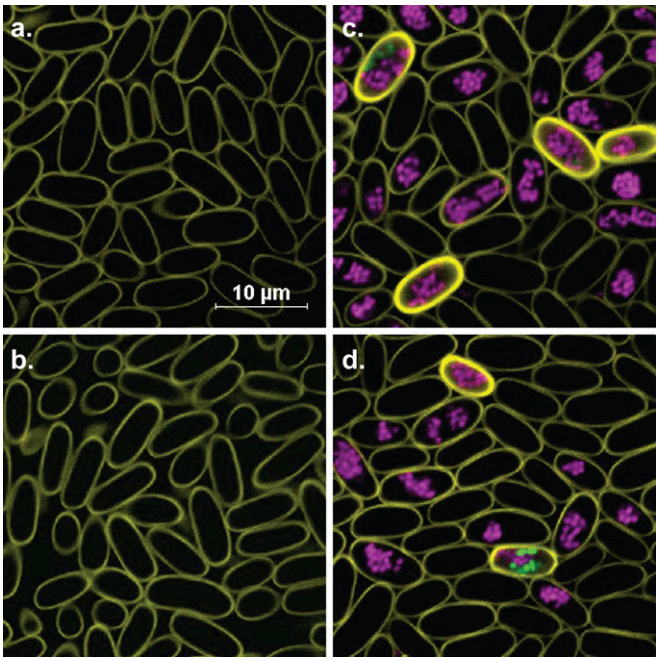

Fig. 5. *Burkholderia* and *K. pneumoniae* can be visualized inside spores after fruiting. (*A* and *B*) *Burkholderia*-negative spores from a tetracycline-treated farmer clone QS70 (*A*) and a nonfarmer QS9 (*B*) after growth on *K. pneumoniae*-GFP and stained with calcofluor-white (pseudocolored yellow) show no evidence of bacterial carriage. (*C* and *D*) Spores from a tetracycline-treated farmer clone QS70 (*C*) and a nonfarmer QS9 (*D*) after growth with 10% B1qs70-RFP (pseudocolored magenta) and 90% *K. pneumoniae*-GFP (green) stained with calcofluor-white (pseudocolored yellow) show B1qs70-RFP and *K. pneumoniae*-GFP inside a portion of the spores.

carry *Burkholderia* through multiple social cycles; (*ii*) produce fewer spores than their noncolonized counterparts under food-rich conditions; (*iii*) produce more spores than their noncolonized counterparts in food-scarce conditions; and (*iv*) pick up and transport secondary bacterial passengers (such as the food *K. pneumoniae*). Second, we show that farmers can be transformed into a nonfarmer-like state by eliminating *Burkholderia* through tetracycline treatment. Thus, like nonfarmers, tetracycline-treated farmers (*i*) no longer carry detectable bacteria through their social cycle; (*ii*) produce more spores in food-rich conditions; and (*iii*) produce fewer spores in food-scarce conditions. These results contrast with our previous study, which found that antibiotic-treated farmers picked up and carried food bacteria right away (24). The earlier study and the current one differ in that in the earlier study we treated the clones with streptomycin/ampicillin rather than tetracycline and we fed the amoebas with an *Escherichia coli* strain rather than *K. pneumoniae*. Clearly there are complexities in the system that we have not fully explored. In addition to *Burkholderia*, we find that other bacterial species can induce moderate levels of bacterial carriage, suggesting that a diversity of soil microbes may be capable of associating with *D. discoideum* with differing degrees of stability.

Despite many phenotypic similarities between *Burkholderia*-colonized nonfarmers and wild farmers, we have retained the farmer vs. nonfarmer terminology in this study to distinguish them according to their original status. That *Burkholderia* causes even nonfarmer *D. discoideum* to carry both *Burkholderia* and food bacteria does not necessarily imply that colonized nonfarmers instantly express all the same characteristics as wild farmers. In future studies, it will be informative to determine whether other farmer-associated traits, such as migration reduction or resilience to specific small molecules, are lost or gained according to *Burkholderia* colonization status. Here, we find that *Burkholderia* colonization results in a fitness tradeoff between spore productivity in different environments. For some *Burkholderia* isolates (particularly B2-tested isolates), infection initially may have more deleterious consequences for naive *D. discoideum* hosts than for evolved hosts. These differences include a greater decline in spore production (Fig. 3*A*), as is consistent with earlier findings that coculturing farmers with nonfarmers decreases nonfarmer spore production in a dose-dependent manner and that some or all of this effect is due to *Burkholderia* (16). Indeed, in the short run, symbionts may impose costs to their hosts, and the host is selected to compensate for these costs (43). Thus, although we show here that *Burkholderia* can easily initiate carriage, we cannot rule out the possibility that the establishment of a mutually beneficial (or at least less host-deleterious) symbiosis may be a rare event requiring coevolutionary adjustment. It will be interesting to investigate further the extent of differential host responses to *Burkholderia* colonization and whether these responses correlate to different association histories.

The association between *D. discoideum* and *Burkholderia* has important phenotypic consequences for their hosts: decreasing spore productivity under optimal conditions but endowing *D. discoideum* with the novel ability to carry additional bacterial species. The trait of secondary bacterial carriage can be beneficial for the amoebas because they can replenish their food supply when dispersed to food-impoverished environments. This trait serves as an unusual example of how symbiotic associations can endow novel functionality to host organisms (44). If we consider that *Burkholderia* colonization may confer adaptive traits to *D. discoideum*, such as the carriage of bacterial food, it may be reasonable to imagine that these adaptive traits may translate into an even broader form of phenotypic plasticity. For instance, *Burkholderia* could allow an amoeba to pick up a variety of new bacterial partners, each generating unique consequences for the host. For instance, in addition to *Burkholderia*, farmer clone QS161 carries a strain of *P. fluorescens* (Pf2) that produces compounds shown to promote host spore productivity while inhibiting nonhost spore productivity (23). Here, we found that this carried *P. fluorescens* strain did not, by itself, associate stably with nonfarmer *D. discoideum* (Fig. S1). Thus, it is likely that the association between Pf2 with its original host may be dependent on *Burkholderia* cocolonization or on another bacterial isolate that likewise induces bacterial carriage. The additional effects exerted on the amoeba host by bacterial cocolonizers may signify a broader adaptive potential arising from *Burkholderia*-induced secondary bacterial carriage. The abundance, persistence, and effects of diverse secondary bacterial passengers suggest an interesting avenue for future pursuits.

The ability of *Burkholderia* to induce the farming phenomenon in *D. discoideum* is consistent with the versatile and often symbiotic nature of *Burkholderia*. The more than 60 species of *Burkholderia* typically are grouped into two main clusters, the human, plant, and animal pathogens, which include species of the *Burkholderia cepacia* complex, and the nonpathogenic, plant-beneficial-environmental species (42). Plant-beneficial-environmental *Burkholderia* are widely distributed in the rhizosphere and have been commonly found in association with a diversity of plant species as well as with some fungi and insects, potentially serving beneficial functions for these hosts. Furthermore, several species of the pathogenic *Burkholderia cepacia* complex have been shown to infect isolates of the free-living amoeba, *Acanthamoeba* (45).

The association with *D. discoideum* expands the list of organisms that serve as hosts for *Burkholderia* and provides a model system for identifying the mechanisms underlying *Burkholderia*'s broad symbiotic potential. Within *D. discoideum* there have been at least two independent origins (clades B1 and B2) of the farmer *Burkholderia* symbionts. Several lines of evidence suggest that this association is important in the wild—that is, it is both widespread and long-established. First, about a third of *D. discoideum* clones collected from the wild show the farming trait (24), although that number should be tested with further collections. Individuals from both *Burkholderia* clades are widely geographically distributed, because they have been sampled from several states (Table S1). As noted above, the severe harm that is done to nonfarmer clones by some *Burkholderia* suggests (but does not prove) that the hosts of these *Burkholderia* have evolved resistance over evolutionary time. Finally, the evidence suggests that one of the secondary passenger bacteria (*Pseudomonas fluorescens*) evolved edibility and many other changes during its association with *D. discoideum* (23).

One example of *Burkholderia*–eukaryote association that bears a particularly striking similarity to our system is the association of *Burkholderia terrae* strain BS001 with soil fungi (46). BS001 has been shown to migrate along growing fungal hyphae, a process potentially aided by the formation of a biofilm around the fungal tip (46, 47). Similar to the induction of secondary bacterial carriage we see with the *Burkholderia–D. discoideum* interaction, BS001 appears to exert a "helper" effect, inducing the comigration of some nonmigratory bacterial species (48). Like farming in *D. discoideum*, the ability to migrate along growing soil hyphae appears to be shared by other phylogenetically distinct *Burkholderia* isolates, including strains related to *Burkholderia terricola*, *Burkholderia xenovorans*, and *Burkholderia phytofirmans* (49). Interestingly, BS001 also has been found to provide its fungus with protection from antifungal agents, such as *P. fluorescens* metabolites and the protein synthesis inhibitor cyclohexamide (47). Perhaps *Burkholderia* provides analogous protection to *D. discoideum*, offering a potential explanation for the resilience of the host farmer, QS161, to the compounds produced by its carried *P. fluorescens* strain (23).

Farmer-borne *Burkholderia* isolates clearly can replicate outside amoeba hosts, as evidenced by our ability to maintain them under regular culture conditions. However, microscopic examination also suggests that these *Burkholderia* isolates may replicate within *D. discoideum* cells. This growth capacity may underlie the ability of these bacterial partners to be acquired from the environment by new host amoebas and to associate stably with old hosts. This pattern implies a mixed mode of horizontal and vertical transmission, as is typically the case for facultative symbionts. The horizontal spread of *Burkholderia* from one cell to another may be an important strategy for maintaining high colonization rates within a *D. discoideum* population, especially given the observation that not all B1qs70-rfp–colonized spores show evidence of internal bacteria. This observation also may explain why some independent *D. discoideum* lines originating from farmer populations no longer carry bacteria: These lines may have been initiated from a single uninfected spore. Whether different *Burkholderia* isolates have higher internal infectivity rates and what affect such variation has on spore viability and development are interesting questions for further research. Differences in these metrics may highlight the mechanistic underpinnings of *Burkholderia* transmission and the differential fitness cost of distinct *Burkholderia* isolates.

By elucidating the mechanism of *Burkholderia* colonization, we may gain further insight into the system normally used by *D. discoideum* to clear itself of bacterial hitchhikers. How *Burkholderia* evades clearance by *D. discoideum* and allows additional bacterial carriage and how bacteria, once internalized, escape amoeba cells remain unclear. It is possible that *Burkholderia* inhibits amoeba phagocytosis and digestion, thereby allowing any coingested bacterial partners to survive. The detection of bacterial cells outside spores may reflect either bacterial exit after sporulation or extracellular bacterial carriage throughout development. If food bacteria associate with *Burkholderia*-colonized fruiting bodies, what excludes food bacteria from these developing structures in noncolonized clones? The bacterial pathogens *Legionella pneumophila* and *Mycobacterium* species replicate and spread intracellularly within *D. discoideum* host cells by subverting a variety of host functions (50–55). To our knowledge, susceptibility to secondary bacterial carriage has not been observed or specifically analyzed during *D. discoideum* infections with these bacterial pathogens. It will be interesting to determine whether induction of secondary bacterial infections can be a byproduct of other microbial infections and to compare the strategies used by bacterial pathogens and *Burkholderia* farmer isolates for intracellular invasion and exit. Our results further support the idea that amoebas can serve as reservoirs for bacterial pathogens but also may have a less nefarious role in aiding the dispersal of bacterial species that may serve important environmental roles (56).

The *Burkholderia–D. discoideum* symbiosis system has several advantages that provide opportunities to address questions of general importance to our understanding of symbiosis. Our ability to induce and separate associations between *Burkholderia* and *D. discoideum* and potentially bias symbiont transmission toward a more horizontal or vertical modality may allow us to examine how modifications in genotypes, transmission modes, environmental conditions, and selection pressures translate into alterations in phenotype and evolutionary outcomes. Their rapid generation times offer opportunities for experimental evolution, and interesting comparisons are afforded by the two clades of symbiotic *Burkholderia*. Given the experimental tools available for use in *D. discoideum* and *Burkholderia*, we can begin to dissect the molecular components driving this association and its corresponding consequences.

Methods

Culture Conditions. To prepare bacterial cultures for growth *with D. discoideum*, we resuspended stationary-phase bacteria grown on SM/5 agar medium [2 g glucose (Fisher Scientific), 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$ (Fisher Scientific), 1.9 g $KHPO_4$ (Sigma-Aldrich), 1 g K_2HPO_5 (Fisher Scientific), and 15 g agar (Fisher Scientific) per liter] at room temperature in KK2 [2.25 g KH_2PO_4 (Sigma-Aldrich) and 0.67 g K_2HPO_4 (Fisher Scientific) per liter]. We determined the initial OD_{600} of each bacterial suspension using an Eppendorf BioPhotometer and diluted with KK2 buffer to a final density of 2. Alternative concentrations (10 or 0.1%) of indicated bacterial isolates were made by mixing the appropriate volume of the indicated bacterial suspension with a suspension of *K. pneumoniae* (both preset to an OD_{600} of 2). All *D. discoideum* isolates were resuscitated from glycerol-frozen spore stocks by plating spores on SM/5 agar medium with 200 μL of *K. pneumoniae* OD_{600} of 2 ($\sim 5 \times 10^8$ cells). Spores from these plates were used subsequently to initiate experimental assays. Table S2 lists all the *D. discoideum* clones used for this study.

Detection of Bacteria in *D. discoideum* Sori (Spot Test Assay). We determined presence of culturable bacteria in the sori of fruited *D. discoideum* clones as previously described (24). Briefly, sorus contents from developed *D. discoideum* fruiting bodies were collected with a 10-μL filter pipette tip, transferred to an SM/5 plate, and incubated at room temperature for up to 2 wk. Ten random sori were sampled per clone, and the presence of bacterial growth per sorus was used as an indication of farming status/bacterial colonization.

Bacterial Isolation. We isolated farmer-associated bacteria by spotting farmer sorus contents on SM/5 agar medium [*Methods*, *Detection of Bacteria in D. discoideum Sori (Spot Test Assay)*] and incubating for up to 1 wk at room temperature. Pure colonies from these spots were generated by restreaking bacteria on SM/5 agar medium up to three times. Once isolates were believed to be isogenic, they were identified via 16s rRNA gene sequencing (*Methods*, *PCR Assays*).

***D. discoideum* Exposure to Distinct Bacterial Species.** To expose *D. discoideum* to bacterial species of interest, we plated 10^5 *D. discoideum* spores with 200 μL of the target bacteria [or, for inedible bacteria, a 10%/90% (vol/vol) mixture of target bacteria/*K. pneumoniae*] resuspended in KK2 to an OD_{600} of 2 onto SM/5 plates. For *Burkholderia* species, 200 μL of 10% *Burkholderia*, $\sim 3.8 \times 10^7$ cells, were plated. After 7 d incubation at room temperature, sorus contents from developed fruiting bodies were tested for bacterial carriage using the spot test assay.

***D. discoideum* Serial Transfer.** To determine the stability of bacterial carriage, we grew 10^5 *D. discoideum* spores from six nonfarmer clones, six farmer clones, and six tetracycline-treated farmer clones with 10% of the indicated bacterial strain mixed with 90% *K. pneumoniae* (transfer 1). For subsequent transfers, 10^5 spores produced from the previous pass (after incubation for 7 d at room temperature) were grown with *K. pneumoniae* only. We determined the percentage of bacterium-positive sori at each pass by performing the spot test assay.

***Burkholderia* Colonization.** To precolonize *D. discoideum* clones with *Burkholderia* isolates, we grew them with the indicated concentration of *Burkholderia* mixed with *K. pneumoniae* in KK2 at an OD_{600} of 2 (mixture set at time of plating). We confirmed successful colonization by performing a spot test assay; clones that produced >90% bacteria-positive sori were considered successfully colonized. We harvested spores from these plates to initiate any assay using precolonized *D. discoideum* clones.

PCR Assays. All PCRs were performed using 1× PCR buffer without $MgCl_2$: 1.5 mM $MgCl_2$, 10 mM $dNTP^s$, 3 μL DNA, 1 U AmpliTaq DNA polymerase (Applied Biosystems), and 0.4 mM of the forward and reverse primers. The reaction was amplified using a touchdown PCR protocol starting with denaturation for 3 min at 95 °C, followed by 15 cycles at 94 °C for 1 min, 63 °C for 1 min decreasing by 0.1 °C per cycle, and 72 °C for 1 min, then cycled for 10 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final extension at 72 °C for 1 min.

For 16s rRNA sequencing for bacterial identification and phylogeny, we extracted bacterial DNA using a Qiagen DNAeasy blood and tissue kit following the manufacturer's protocol. We amplified 16S rRNA using the forward primer CGGCCCAGACTCCTACGGGAGGCAGCAG and the reverse primer GCGTGGACTACCAGGGTATCTAATCC. After amplification, we cleaned

PCR products with ExoSap and sequenced using Applied Biosystems BigDye v1.1, v3.1. PCR fragments generated were sequenced at the Biology DNA Sequencing Facility at the Danforth campus of Washington University in St. Louis. The closest GenBank relatives for each isolate were determined by aligning resolved sequences against the curated 16S ribosomal RNA sequence database in the National Center for Biotechnology Information database. These sequences have been deposited in GenBank (accession numbers KR607499–KR607513).

To detect bacteria in *D. discoideum* sori, we collected the contents of 10 sori from each *D. discoideum* clone and extracted DNA using a Chelex/proteinase K protocol. To detect bacterial DNA, we used our forward and reverse 16s rRNA primers (see above). To detect eukaryotic DNA, we used the forward primer AACCTGGTTGATCCTGCCAGT and the reverse primer TCGAGGTCTCGTCCGTTATC to amplify 17S rRNA. To detect *Burkholderia* DNA, we used the *Burkholderia*-specific forward and reverse primers CTGCGAAAGCCGGAT and TGCCATACTCTAGCYYGC (57). To detect *Klebsiella* DNA, we used the *Klebsiella*-specific forward and reverse primers ATTTGAAGAGGTTGCAAACGAT and CCGAAGATGTTTCACTTCTGATT (58). After PCR amplification, we ran 10 μL of each PCR on a 1% agarose gel with 0.1% ethidium bromide to image the presence or absence of bands.

Spore Production Assays. For all spore count assays, 10^5 spores were used at plating, and total spores were determined 7 d after plating. To harvest spores, we flooded each plate with 5–10 mL KK2 + 0.1% Nonidet P-40 and collected the entire surface contents into 15-mL Falcon tubes. We then diluted our samples in KK2 and counted spores on a hemocytometer. Spore counts were replicated three times for each clone by condition. For counts comparing colonized and uncolonized nonfarmer spore productivity under food-rich conditions, spores from six uncolonized nonfarmers, six *Burkholderia*-colonized nonfarmers, and four host farmers (either pregrown on *K. pneumoniae* alone or reexposed to their original *Burkholderia* isolate via pregrowth with 10% or 0.1% *Burkholderia* mixed with 90% or 99.9% *K. pneumoniae*) were plated with 200 μL of *K. pneumoniae* at an OD_{600} of 2 onto SM/5 nutrient medium. For counts comparing untreated and tetracycline-treated farmer spore productivity under food-rich conditions, spores from four untreated farmers and their four tetracycline-pretreated counterparts (*Methods*, *Tetracycline Treatment of Farmer* D. discoideum) were plated with 200 μL of *K. pneumoniae* at an OD_{600} of 2 onto SM/5 nutrient medium. For food-poor conditions, clones were pretreated in the identical fashion as described for food-rich conditions; however, spores were plated with 200 μL of heat-killed (heated for 30 min at 80 °C) *K. pneumoniae* at an OD_{600} of 6 onto SM/5 nutrient medium.

Tetracycline Treatment of Farmer *D. discoideum*. To eliminate carried *Burkholderia* from wild farmer clones, we plated 1–10 spores of each farmer clone with 500 μL of *K. pneumoniae* resuspended in KK2 to an OD_{600} of 100 onto SM/5 agar plates with 30 μg/mL tetracycline and incubated them at room temperature. We replated 1–10 spores produced on these plates under the same conditions for a second pass on tetracycline. Spores produced on the second tetracycline plate (1×10^5) then were plated onto SM/5 with 200 μL *K. pneumoniae* (OD_{600} 2). We confirmed the loss of bacterial carriage and *Burkholderia* colonization using the spot test assay and PCR analysis of eubacteria, *Burkholderia*, and *K. pneumoniae* amplification in *D. discoideum* sori.

Phylogeny Construction. Sequences were trimmed to 400 nt, cleaned, and aligned using Geneious version 5 (Biomatters; www.geneious.com). Taxa selection for the phylogeny included all 31 defined plant-beneficial-environmental species (16) and a few representatives from the pathogenic clade. The trees were rooted using the pathogenic species *B. pseudomallei* as an outgroup. Phylogenetic reconstructions were done using both distance-based (neighbor-joining) and Bayesian analyses. Distance-based trees were reconstructed using the program MEGA (59) using the Tamura-nei model of nucleotide substitution and 1,000 bootstrap replicates. Bayesian analyses were run in Mr. Bayes version 3.1.2 (60) using the general time-reversible model and were run for 10 million generations. Four Monte Carlo chains were run; trees were collected every 100 generations, with the first 10,000 discarded as burn-in.

Microscopy. An RFP-labeled version of B1qs70 was constructed by performing a triparental mating procedure using the *E. coli* helper strain E1354 (pTNS3-asd_{Ec}) and the *E. coli* donor strain E2072 with pmini-Tn7-gat-P1-rfp as previously described (61, 62). RFP-positive *Burkholderia* conjugants were confirmed using *Burkholderia*-specific PCR. A GFP-labeled version of *K. pneumoniae* (strain ID DBS0349837) was kindly provided by Dictybase and the Dicty Stock Center (63). QS9 and the tetracycline-treated B1qs70 host farmer were colonized by B1qs70-RFP by plating 10^5 spores with 200 μL of a 10/90 (vol/vol) mixture of B1qs70-RFP/*K. pneumoniae*-GFP in KK2 at an OD_{600} of 2. Control samples were plated with *K. pneumoniae*-GFP only. Four to seven days after plating, spores were harvested from fruiting bodies and incubated in 80 μL KK2 with 10 μL of 10% calcofluor for 5–10 min before imaging on a Nikon A1Si laser-scanning confocal microscope using the 100× objective and Nikon Elements software.

Statistical Analysis. We analyzed all data using *R* v3.0.1. We tested the statistical significance of model parameters using likelihood ratio tests on full models fit with and without the parameter of interest. For spore productivity data which contained data with heterogeneity of variances, we fit linear mixed effects models to spore count data using the lme command in the nlme package. Additional variance terms were incorporated as weighted random effects to model the variance structure, avoiding the need for data transformation. Maximum likelihood estimation was used to determine the fixed effects structure. In all cases, clone identity was modeled as a random effect. For spore productivity of colonized versus uncolonized nonfarmers in food-rich and food-scarce conditions, we modeled colonization status as a fixed effect. For farmer versus nonfarmer comparisons we modeled farming status as a fixed effect. For *Burkholderia* dosage comparisons, we modeled dosage as a fixed effect. For spore productivity of *D. discoideum* after exposure to distinct *Burkholderia* clades, we modeled clade identity as a fixed effect. For spore productivity of tetracycline-treated (i.e., cured) versus untreated farmers, we modeled clone identity as a random effect and tetracycline treatment as a fixed effect. All data files have been submitted to Dryad (https://doi.org/10.5061/dryad.pb2sq).

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