Symbiosis
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Which phenotypic traits of *Dictyostelium discoideum* farmers are conferred by their bacterial symbionts?

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Abstract The bacterial partners in symbiotic relationships with eukaryotes can have a powerful effect on the phenotypic traits of the host. Here we explore this issue using a simple model eukaryote, *Dictyostelium discoideum*, and its facultative bacterial symbionts. Some clones of the social amoeba *D. discoideum*, called farmers, maintain symbiotic relationships with certain species of bacteria while other clones, called non-farmers, do not. *D. discoideum* farmer clones that carry bacterial symbionts in the genus *Burkholderia* have four distinct traits associated with the farming symbiosis: i) short slug migration distances, ii) symbiont transport, iii) prudent harvesting of bacteria, and iv) resistance to toxicity of the farmer-associated bacteria. These traits, with their advantages and disadvantages, could be either conferred by bacterial symbionts, or intrinsic to the amoebae that have been colonized by bacteria. We compared five farmers and five non-farmers, both with and without farmer-associated bacteria, to disentangle the direct effects of bacteria from those intrinsic to the host. We found that short migration distance and symbiont transport are bacterially conferred traits. Prudent harvesting seems to represent a trait influenced by bacteria but not entirely controlled by them. Resistance to farmer-associated *Burkholderia* is present whether or not the farmer clone is currently carrying it or has been cured and newly exposed. Taken together, these data suggest that the association between host farmer and *Burkholderia* is not recent.

Keywords *Dictyostelium* · *Burkholderia* · Farming symbiosis · Phenotypic traits

1 Introduction

The genes of individuals build phenotypes, usually construed as traits of those individuals' own bodies. However, an individual's genes can also have extended phenotypes that reach outside its own body, such as a wasp's nest or a beaver's dam (Dawkins 1982). Such phenotypes can even extend into the bodies of others. This kind of interaction is perhaps most pervasive in symbioses, where two partners live together and have extensive opportunities to affect each other. Many of these effects in symbioses are beneficial or mutualistic but others, even with mutualisms, may be harmful or manipulative. Teasing apart which partner causes which traits is an important part of understanding any symbiosis.

Symbiotic relationships between bacteria and eukaryotes are common and can involve multiple symbiotic bacteria in the same host. These symbiotic microbial associates can have striking effects on host organism phenotypes (Douglas 1994; Dale and Moran 2006; Moran 2006, 2007). Traits related to appearance are sometimes the easiest to observe but phenotypic traits can also include behavior, metabolism, survival, development, reproduction, and defense.

Phenotypic changes in hosts can occur as a result of interactions with symbionts. Examples found in some of the well-known symbioses include squid-*Vibrio* symbiosis (Nyholm and McFall-Ngai 2004) where the symbiont produces light

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used as counter-illumination by the squid to avoid predators, plant-rhizobia symbiosis (Friesen et al. 2011) where the fungus increases plant mass, and aphid-*Buchnera* symbiosis (Oliver et al. 2010) where the symbiont provides essential nutrients missing in the aphid diet. Additionally the facultative symbionts of the pea aphid affect many traits. Some examples include the bacterial symbiont, *Hamiltonella defensa* that confers resistance to aphids from parasitic wasps (Oliver et al. 2005), the symbiont *Regiella insecticola* that protects aphids from fungal pathogens (Scarborough et al. 2005), and the symbionts *Serratia symbiotica* and *H. defensa* that confer tolerance to high temperatures (Russell and Moran 2006). For any traits associated with symbiosis, it is important to know how these phenotypes arise: whether they are conferred by the symbionts, or are intrinsic characteristics of the host that carries the symbiont. This is most easily evaluated with a facultative symbiosis where host carriers and non-carriers can be compared with and without the symbiont. This type of experimental comparison is not always feasible if the symbionts or their hosts are not easily cultured or cured, particularly if the host/symbiont relationship is obligate. Therefore a simple model system with a culturable host and a few culturable symbionts is ideal for understanding the complex interactions of hosts and their microbial associates.

In this study, we explore the nature of traits associated with an agricultural symbiosis in the model eukaryote *Dictyostelium discoideum*. Certain clones of this soil-dwelling social amoeba beneficially interact with several bacteria species for both defense and as food sources (Brock et al. 2011, 2013; Stallforth et al. 2013). In this proto-farming symbiosis, clones transport food and non-food defensive bacteria to new locations where they grow, though without further known cultivation. Then these clones exhibit a form of prudent harvesting by not eating all available food but instead saving some for transport to the next new location. For this reason we call wild isolates of *D. discoideum* that carry bacteria, farmers, and wild isolates that do not, non-farmers.

Wild *D. discoideum* are found in soil and leaf litter where the vegetative, solitary stage of *D. discoideum* consists of individual amoebae consuming prey bacteria (Kessin 2001). Upon starvation, amoebae enter a social, multicellular stage and aggregate by the thousands to form a multicellular slug, which can migrate in search of new food sources. Eventually the slug terminally differentiates into a fruiting body containing about 80 % reproductive spores and about 20 % dead stalk cells that lift up the spore mass (sorus) to aid in dispersal. Recently we determined that bacterial carriage and stable association with bacterial symbionts is initiated by farmer-associated *Burkholderia* (DiSalvo et al. 2015). Non-farmer clones infected with *Burkholderia* display a core trait of farming, carriage of bacteria, retaining association with bacteria through multiple rounds of growth after initial colonization. Additionally, we generated a phylogeny of farmer-associated *Burkholderia* bacteria based on 16S rRNA. It is clear that the associates of *D. discoideum* are in the environmental clade of *Burkholderia* species and within it form two main distinct clades, which we here call simply Clade 1 and Clade 2. In this study we focus on Clade 2.

Clade 2 *Burkholderia* have been shown to help the farmers not only by causing carriage of food and non-food bacteria, but also by limiting the growth of non-farmer clones and by promoting farmer growth (Brock et al. 2013). However, carrying *Burkholderia* Clade 2 bacteria has disadvantages. Notably, non-farmer amoebae cannot proliferate on *Burkholderia* Clade 2 alone because it is not a food (Brock et al. 2013). At lower concentrations of *Burkholderia* Clade 2 bacteria mixed with *Klebsiella pneumoniae*, a common food bacterium for *D. discoideum*, non-farmers still experience great losses in productivity while farmers remain relatively unharmed. Here we ask if curing farmers with antibiotics to remove the farmer-associated bacteria, rendering the cured farmers similar to naïve non-farmers could change trait expression. A change in trait expression of a cured farmer relative to an uncured farmer still carrying its farmer-associated bacteria could suggest that the trait is bacterially-associated. On the contrary, if a cured farmer behaves similarly to an uncured farmer, this would suggest that the trait was intrinsic to the host. Indeed, just as seen in host/symbiont interactions for many other systems, we found *Burkholderia* Clade 2 symbionts are responsible for some of our observed phenotypic traits for farmers. However, we were also able to show experimentally that some farmer phenotypic traits are not associated with the symbiont but are characteristics of *D. discoideum* farmers. These adaptations generate new phenotypic diversity because both cured and uncured farmers remain similar for some traits yet differ significantly from naïve non-farmers.

2 Material and methods

2.1 Clones and culture conditions

We used a population of ten clones: five non-farmers (QS1, QS6, QS9, QS17, QS18,) and five farmers (QS11, QS21, QS22, QS23, QS155) isolated from Mountain Lake Biological Station, VA (N 37° 21′, W 80° 31′). The five farmer clones each carry individually isolated non-food bacteria from *Burkholderia* Clade 2 identified by 16S ribosomal RNA as well as food bacteria such as *Klebsiella ssp*. (Supplementary Table 1). We grew clones on nutrient agar plates (2 g peptone, 0.2 g yeast extract, 2 g glucose, 1.9 g KH_2PO_4, 1.3 g K_2HPO_4, 0.2 g $MgSO_4$ anhydrous and 17 g of agar per 1 L of H_2O) in conjunction with *Klebsiella pneumoniae* grown at 21 °C as a food source for the *D. discoideum*. To prepare bacteria-free farmer clones, we plated spores from all ten clones on nutrient agar plates supplemented with antibiotics (0.1 g ampicillin,

0.3 g streptomycin sulphate per liter) which will kill *Burkholderia* Clade 2 and *K. pneumoniae* if present while leaving the amoebae unharmed, using dead *K. pneumoniae* as food. We collected spores from these cured clones after fruiting and passed them through two rounds of growth on live *K. pneumoniae* as above. We verified that these cured clones were bacteria free by the spotting assay and by PCR (see below for assay details). Results of the PCR are displayed in Supplementary Figure 1.

2.2 Bacteria presence or absence assays

Spotting assay Seven days post plating of *D. discoideum* spores and bacteria on nutrient agar plates, we set up spotting assays in order to test for bacterial presence. For the spotting assay we collected ten random individual sori from fruiting bodies using a dissecting scope and filtered pipet tip. These sori were individually transferred to a nutrient agar bacterial plate by touching the tip to the surface of the agar (Fig. 2). After 5 to 7 days, the spotting assays were scored for presence or absence of bacteria colonies and fruiting bodies for each of the ten spotted sori for each clone.

PCR and sequencing assay To determine *Burkholderia* presence or absence in *D. discoideum* sori, we collected sori from 8 to 10 fruiting bodies from each clone and extracted DNA using a Chelex/Proteinase K protocol (Biorad). The PCR amplification was done using a Gene Amp kit from Applied Biosystems (Roche). We used a primer set specific for *Burkholderia* amplification (Salles et al. 2002). We used forward sequence 5′-CTG CGA AAG CCG GAT-3′ and reverse sequence 5′-TGC CAT ACT CTA GCY TGC-3′. 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 10 cycles at 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 1 min with a final extension cycle at 72 °C for 1 min. We ran 10 μl of each PCR reaction on a 1 % agarose gel +0.1 % ethidium bromide in TBE buffer (10.8 g Tris base, 5.5 g Boric acid, 0.93 g Na_2 EDTA per 1 L of H_2O) to image the presence or absence of PCR fragments.

2.3 Migration assay

Migration is the movement of multicellular slugs of *D. discoideum* towards light. To set up plates for slug migration, we prepared a concentrated suspension of stationary phase *K. pneumoniae*. We prepared the bacteria by centrifuging an overnight culture of *K. pneumoniae* at 10,000 g for 5 min at 10 °C. After centrifugation, we suspended the pellet in non-nutrient buffer (2.25 g KH_2PO_4 and 0.67 g K_2HPO_4 per liter of H_2O). To use a standard number of bacteria, we used optical density which we determined using a BioPhotometer and diluted the solution to a density A_{600} of 75.0 in non-nutrient buffer. We collected spores from each test clone, uncured and cured with antibiotics, in non-nutrient buffer and counted dilutions using a hemacytometer. In a 1.5 ml tube, we mixed 200 μL of the prepared *K. pneumoniae* and 5×10^6 spores. We plated the mixture in a straight line across a non-nutrient agar plate (0.0356 g of Na_2HPO_4, 0.198 g KH_2PO_4 and 17 g of agar per liter of ddH_2O). After the mixture dried, we marked zones of 1.5 cm in width across the bottom of the agar plate from the original line where the spores were placed in order to measure the distance slugs travelled after migration. Next, we wrapped the plates in aluminum foil and added a small hole directly opposite the line of spores. The plates were placed on the lab bench at room temperature and under a direct light. These conditions allow for spore hatching, proliferation of amoebae, and formation of slugs. After the amoebae consume all of the food bacteria, starvation initiates the social stage where slugs form and migrate phototropically across the agar plate towards the pinhole of light. We allowed the slugs to migrate for about 80 h before the foil was removed. When the plates were unwrapped, we first photographed the plates then we placed them under direct light on the bench so that the slugs would form fruiting bodies, the spore dispersal structure. Placing slugs under direct light causes slugs to stop migrating so the forming fruiting bodies will be in roughly the same location as the slugs when photographed. Using the photographs, we counted the number of slugs formed in each 1.5 cm zone and used this data to calculate the average distance each slug travelled over the 80 h period. Then 5 days after unwrapping the plates, we performed a spotting assay selecting the farthest migrating fruiting bodies. Three days later, the spotting assays were scored and the data collected. We performed two replicates.

2.4 Prudent harvesting assay

We used a bacteria usage assay to determine if there is a difference in the way cured clones and uncured clones use bacteria. The bacteria usage assay was performed using the same population of five non-farmer clones, five cured non-farmer clones, five farmer clones, and five cured farmer clones. We collected spores from these clones and spotted four 30 μl spots containing 3×10^4 spores mixed with live *K. pneumoniae* on nutrient agar plates. For a control, we also spotted 30 μL of *K. pneumoniae* with no added spores on nutrient agar plates. We collected data on day 7 after plating by which time *Dictyostelium* fruiting is completed and spots of bacteria and/or *Dictyostelium* still remain separate from each other. In order to test for bacterial and spore density, we used a sterilized loop to collect all growth individually from each of two separate spots on the plate. Contents of each spot were placed in individual 1.5 ml Eppendorf tube with 1 ml of

non-nutrient buffer. We vortexed each mixture and removed 10 μl for counting *D. discoideum* spores with dilution using a hemacytometer and microscope. Next, we completed differential centrifugation to separate hatched amoebae, spores, and bacteria by spinning at 300G to pellet the amoebae and spores. After, we removed the supernatant containing the bacteria and determined the bacterial absorbance (A_{600}) using a BioPhotometer (Eppendorf, NY). We averaged the data collected from the two spots for each clone and we performed one replicate.

2.5 Assay for resistance to *Burkholderia* Clade 2

We prepared the *Burkholderia* Clade 2 bacteria by growing up nutrient agar plates of the bacteria that was isolated from farmer clone QS11. Then, we collected the *Burkholderia* Clade 2 using a sterile loop and suspended the bacteria in starvation buffer. We also collected and prepared a similar suspension of *K. pneumoniae.* We determined the optical density of each bacteria sample using the BioPhotomer (Eppendorf, NY) and diluted the solution to an A_{600} of 1.5 in starvation buffer. In order to prepare plates for spore production in *Burkholderia* Clade 2, we set up a suspension of 95 % *K. pneumoniae* and 5 % of *Burkholderia* Clade 2 for the initial plating proportion. We collected spores of antibiotic cured farmer and non-farmer clones in non-nutrient buffer and counted a dilution using a hemacytometer. We plated 2×10^5 spores plus 200 μL of either the 5 % *Burkholderia* Clade 2 mixture or 100 % *K. pneumoniae* for each clone per nutrient agar plate in duplicate. We collected whole plate spores 5 days after fruiting bodies formed by using starvation buffer supplemented with 0.1 % NP-40 alternative (Calbiochem, CA). We counted the spores for each clone and averaged the duplicates. We performed two replicates.

2.6 Statistical analyses

To analyze our data, we used a generalized linear mixed model with fixed effects (farmer and non-farmer) in addition to a random affect (clone) for all tests except symbiont transport. We used less than or equal to 0.05 as the cut-off for statistical significance, and we used a post hoc Tukey HSD test to correct for multiple comparisons with significant differences indicated by different letters. Standard error and F-statistics were Kenward Rogers corrected in order to approximate degrees of freedom using variances and correlations in the collected data (Kenward and Roger 1997). We used SAS software (Version 9-2 of the SAS System for Windows, Copyright 2002–2003, SAS Institute Inc.). For the symbiont transport test, where the data were non-normal and the variances heterogeneous, we used a generalized linear mixed model fit by maximum likelihood with binomial error distribution, implemented in RStudio version 0.99.484 (RStudio Team 2015) using the BLME package (Dorie 2015).

3 Results

In these experiments, we tested four traits associated with the farming symbiosis (shorter migration distances, symbiont transport, prudent harvesting of bacteria, and resistance to the toxicity of the farmer-associated bacteria) to see if they stay the same or change when farmer bacteria are removed by antibiotics (curing). If they stay the same, it is consistent with the trait being caused by the host farmers. If they change to the trait of non-farmers that do not carry bacteria (uncured or cured) then the trait would appear to be caused by presence of the bacteria.

Figure 1 illustrates expected outcomes under different control hypotheses for comparison with actual results in later figures. *Burkholderia* presence is indicated by solid bars and *Burkholderia* absence by hatched bars, highlighting the fact that three of the four treatments lack symbionts. If the trait difference is caused by the host's farmer status, then the two farmer bars (left) should differ from the two non-farmer bars (right). If instead, the trait difference is caused by the presence of *Burkholderia* symbionts, the solid bar (host with symbiont) should be different from the three hatched bars (hosts without symbiont). The key is whether the cured farmer bar (red hatched) is like the uncured farmers or the non-farmers. If it

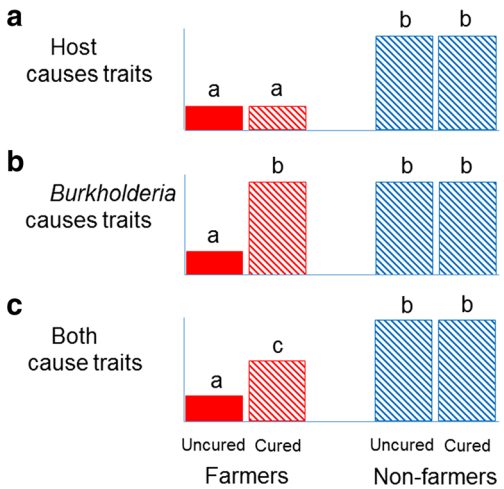

Fig. 1 Expected outcomes under different trait hypotheses. The key issue is whether the trait value of farmers cured of their *Burkholderia* bacteria still resemble uncured farmers, or now resemble non-farmers (cured and non-cured). Treatment groups are labeled and hatching is used to highlight the three treatments that are *Burkholderia* free. Host causing the trait is supported when cured farmers more resemble uncured farmers (left two bars differ from right two bars; panel **a**). *Burkholderia* causing the trait is supported if they more resemble non-farmers (all *Burkholderia* -free hatched bars differ from the solid bar with *Burkholderia*; panel **b**). Both the host farmer and *Burkholderia* causing the trait are supported by an intermediate result (panel **c**)

is intermediate between them, then we would conclude that both host status and bacterial presence contribute to the trait. Note that the "cured" non-farmers are really just a control for unlikely antibiotic effects; because non-farmers have no bacteria to cure, the cured non-farmers should act the same as the uncured ones. As expected, the cured and uncured non-farmers results are in fact indistinguishable in all of our experiments.

We used five *D. discoideum* farmers found associated with *Burkholderia* Clade 2 and five *D. discoideum* non-farmer clones. We cured these five farmer clones with antibiotics to remove their host-associated bacteria. We cured five non-farmer clones identically as a control for the antibiotic treatment. In total, we tested 20 clones for each assay (five farmer clones, cured and uncured, and five non-farmer clones, cured and uncured). For the resistance to *Burkholderia* Clade 2 assay, we only tested the 10 cured clones since we have previously reported uncured farmers are resistant to harm from *Burkholderia* Clade 2 symbionts while uncured non-farmers suffer great harm (Brock et al. 2013). We verified the presence or absence of bacteria in all 20 of the *D. discoideum* test clones by spotting ten fruiting body sori individually on nutrient agar plates to check for bacterial growth. A cartoon of the spotting assay and representative examples of spot tests are shown in Fig. 2. We found no bacteria growth (negative spot tests) in the cured clones and no bacteria growth in the five non-farmer clones. By contrast, all wild farmers carried bacteria as indicated by their positive spot tests. Additionally, we used PCR and *Burkholderia*-specific primers on template DNA prepared from sori from each clone and found the same results (Supplementary Figure 1). DNA from all cured and uncured non-farmer sori as well as cured farmer sori were negative for *Burkholderia* 16S DNA, while all five uncured farmers were positive.

3.1 Slug migration distance

We previously demonstrated that multicellular slugs of farmer clones migrate shorter distances than non-farmers (Brock et al. 2011). Here we asked if farmer-associated bacteria are inhibiting the migration of farmers. For the migration assay, we induced the slug formation stage and allowed them to migrate towards light for about 80 h. We tested whether migration distance varies by farmer status and/or with antibiotics to remove farmer-associated bacteria by performing a two-way analysis of variance. We found a strongly significant effect of farmer status, curing status and interaction between farmer/non-farmer status and curing status (Mixed model analysis of variance: Farmer $F_{1,8} = 5.89$ $p = 0.0414$; Cured $F_{1,8} = 41.85$ $p = 0.0002$; Farmer*Cured $F_{1,8} = 29.27$ $p = 0.0006$).

However, the treatment effects in our generalized linear mixed model do not directly address our real question of who causes the trait differences. Instead we need to look at particular contrasts, both in this section and those that follow. We confirmed, as previously reported, that non-farmer clones migrated farther than farmers (Brock et al. 2011). We show that removing the farmer-associated bacteria allowed the cured farmers to migrate significantly farther than uncured farmers and migrate distances similar to the non-farmers both cured and uncured (Fig. 3). Our results therefore demonstrate that migration distance is a bacterially conferred trait since removing the farmer-associated bacteria resulted in the cured farmers behaving like non-farmers.

3.2 Symbiont transport trait

Recent work showed that infecting non-farmers with farmer-associated *Burkholderia* isolates caused carriage of food and

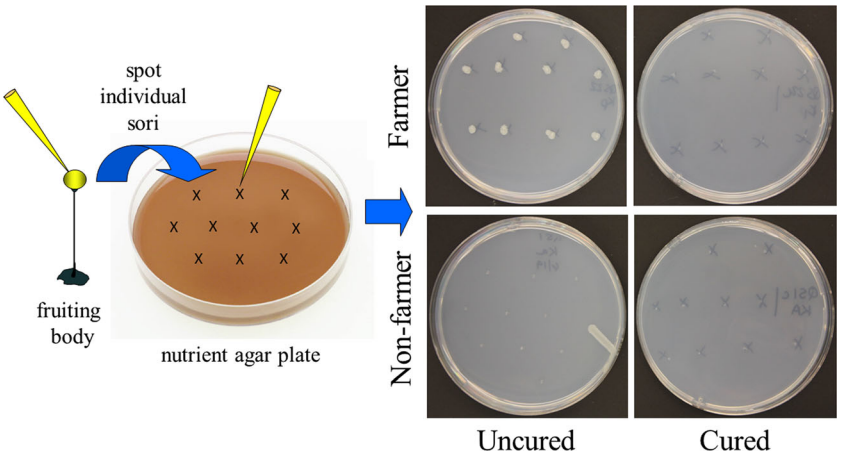

Fig. 2 Antibiotic treatment eliminates bacteria carried in the sori of *D. discoideum* farmer clones. Ten individual, random sori from five farmers and five non-farmers untreated and treated with antibiotics were spotted on nutrient agar plates to visualize bacteria presence or absence in the sorus shown by cartoon representation on the left. On the right are representative examples of one uncured and cured farmer and non-farmer. White spots present in the uncured farmer image are bacterial growth from bacteria present in the individually plated sori

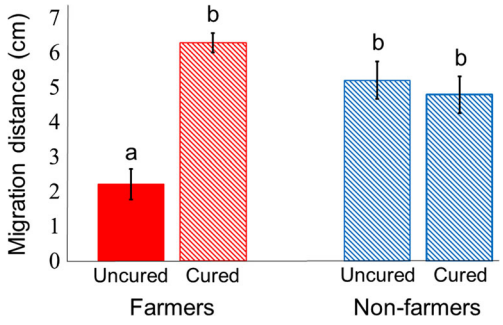

Fig. 3 Cured farmers migrate farther than uncured farmers. We measured the distance slugs migrated using both uncured and cured farmers and non-farmers. Cured clones were treated with antibiotics to remove farmer-associated bacteria, processing non-farmers the same as a control. Cured farmers increase migration distance suggesting a bacterially conferred trait. We used a post hoc Tukey HSD test to correct for multiple comparisons and significant differences are indicated by different *letters*. *Error bars* equal s.e.m.; N equals 20

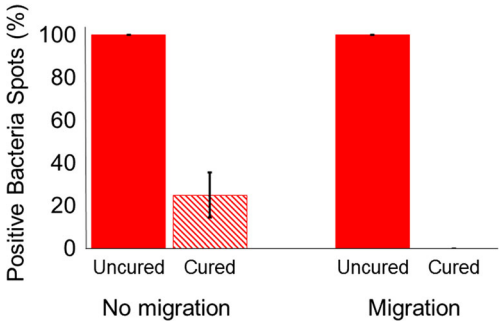

Fig. 4 Migration has no effect on ability of uncured farmers to carry bacteria through the social stage. To determine what are the predictors of bacterial carriage, we tested for positive bacteria presence in the sori of ten random fruiting bodies from clones allowed to migrate or form fruiting bodies without migration using five each uncured and cured farmers. We asked if antibiotic treatment to remove farmer-associated *Burkholderia* Clade 2 bacteria and/or migration affect carriage of bacteria. We compared two models and found antibiotic treatment significantly predicts bacteria carriage in farmers ($p < 0.001$) while migration does not ($p = 0.539$). Error bars equal s.e.m.; N equals 10 with two biological replicates

Burkholderia bacteria (DiSalvo et al. 2015). This suggests that a core trait of farming – the carriage of bacteria – is caused by *Burkholderia* rather than *Dictyostelium*. Here we repeat this kind of experiment, but with two migration treatments. Migration away from food bacteria might change the ability of *D. discoideum* farmers to transport bacteria because during the social stage specialized cells function to remove bacterial pathogens and toxins from the presumptive spore population. Also, we have previously published that non-farmers do not carry bacteria and were used here in this experiment as a control (Brock et al. 2011). Therefore we divide the data into farmers and non-farmers from the outset because we have a prior expectation that bacteria carriage will be significantly different between these two groups. Our main question to answer here is "What are the predictors of bacterial carriage for farmers? We used two models to test whether antibiotic treatment to remove farmer bacteria (model 1) and/or migration (model 2) affect carriage.

Comparing our two models we found antibiotic treatment significantly predicts bacteria carriage in farmers ($p < 0.001$) while migration does not ($p = 0.539$) as shown in Fig. 4. We confirmed the prior finding that carrying bacteria is a trait conferred by *Burkholderia*. Neither antibiotic treatment ($p = 0.9393$) or migration ($p = 0.9393$) significantly predict carriage of bacteria in non-farmers either cured or uncured (data not shown). These data support the ability to transport bacteria is a trait that is conferred by the bacterial symbiont.

Model 1 (carriage of bacteria) has an AIC of 20.93415 and model 2 (migration) has an AIC of 60.64796 but when we compared the two models there was no difference in value as predictors of carriage ($p = 1.0$). This may be because all uncured farmers carry bacteria at 100 % regardless of migration of farmers. Curiously, though not significant, we observed a reduction in positive bacteria presence in sori for cured farmers after migration. The small amount of carriage seen in the no-migration treatments may be a different kind of carriage, possibly incidental, because unlike the carriage in farmers with *Burkholderia*, it tends to vanish with migration.

3.3 Prudent harvesting trait: bacteria left unconsumed

We have previously reported that farmer clones of *D. discoideum* do not consume all available food bacteria before entering the social stage and forming fruiting bodies (Brock et al. 2011). Non-farmer clones, on the other hand, consume essentially all available food bacteria. We wanted to know if the cured farmers exhibited behavior similar to uncured farmers or were similar to control cured or uncured non-farmers. To examine this, we spotted a fixed number of spores and *K. pneumoniae* food bacteria in duplicate spots on nutrient agar plates. Bacteria and *Dictyostelium* grew outward from the spot, thus forming a larger colony. On the seventh day, after all bacterial and *Dictyostelium* growth had ceased and *Dictyostelium* fruiting was complete, we measured the concentration of bacteria left uneaten. We tested whether the amount of bacteria left unconsumed before completing the social stage varies by farmer status and/or treatment by performing a two-way analysis of variance. We found strongly significant effects for all main effects and interactions (Mixed model analysis of variance: Farmer $F_{1,16} = 642.78$ $p < 0.0001$; Cured $F_{1,16} = 225.59$ $p < 0.0001$; Farmer*Cured $F_{1,16} = 208.58$ $p < 0.0001$). Again, particular contrasts are more revealing. Uncured farmers leave about half of the potentially available bacteria unconsumed while non farmers, cured or uncured, left far fewer bacteria unconsumed (Fig. 5a). Cured farmers leave an intermediate amount uneaten: significantly less than uncured farmers but significantly more than either

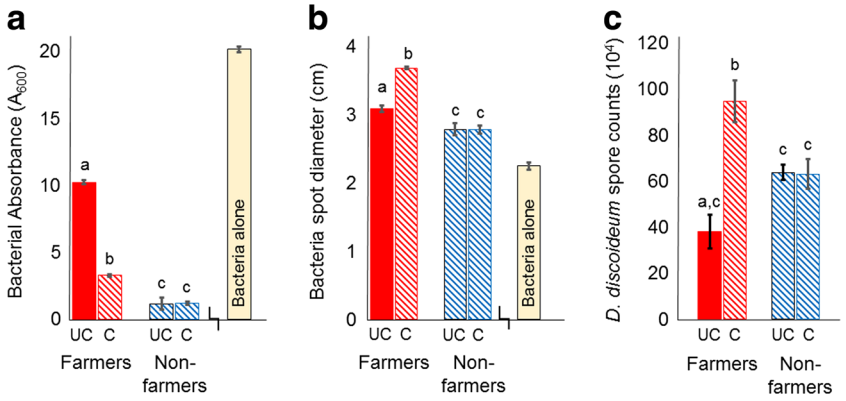

Fig. 5 Cured farmers do not consume all available bacteria, expand farther, and produce more spores than uncured farmers. We tested the effect of removing farmer-associated bacteria on three aspects of the prudent harvesting farmer trait after spotting *Dictyostelium* and food bacteria on nutrient plates and allowing growth to cessation. Effects on the trait could be bacterially conferred, intrinsic to the amoebae, or a combination of the two. **a** The amount of bacteria uneaten. Uncured farmers leave about half of available bacteria uneaten and non-farmers leave very little. Cured farmers leave an intermediate amount uneaten: significantly less than uncured farmers but significantly more than both uncured and cured non-farmers. Both the bacteria and changes in the farmer amoebae affect this trait. **b** The distance colonies were able to expand before cessation of growth. Uncured farmers expand farther than either uncured or cured non-farmers, however cured farmers expand even farther than the uncured farmers. **c** Number of *Dictyostelium* spores produced. Uncured farmers produce fewer spores than non-farmers but cured farmers produce more than all of them. We used a post hoc Tukey HSD test to correct for multiple comparisons and significant differences are indicated by different letters. Error bars equal s.e.m.; N equals 20

cured or uncured non-farmers (Fig. 5a). Our data therefore suggest that amount of bacteria eaten by a farmer clone is neither completely controlled by the host-associated bacteria or completely due to the amoebae but instead is intermediate and both have some influence.

3.4 Prudent harvesting: colony expansion

In the same experiment, we observed that farmers expanded their bacterial colony size compared to bacteria spotted alone. To examine this quantitatively, we investigated colony size for the prudent harvesting trait by measuring and recording the diameter of the bacteria spot 7 days after plating and comparing that diameter to a bacteria-only control to determine if this trait varies by farmer status and/or cured status. We performed a two-way analysis of variance on the data and found strongly significant effects for all main effects and interactions (Mixed model analysis of variance: Farmer $F_{1,16} = 104.96$ $p < 0.0001$; Cured $F_{1,16} = 25.40$ $p = 0.0001$; Farmer*Cured $F_{1,16} = 22.18$ $p = 0.0002$). Note first that all treatments appear to produce larger colony sizes than the food bacterium alone, consistent with either bacteria being moved by amoebas or with amoeba predation delaying the time at which bacteria deplete their resources. Uncured farmers expand significantly farther than the uncured and cured non-farmers but curiously the cured farmers expand significantly farther than all of the others (Fig. 5b). This suggests a more complex form of interaction than represented by the hypotheses in Fig. 1. An alternative hypothesis is that farmers in general are more prudent but that the effect differs when they are grown on mixed populations of *Burkholderia* and *K. pneumoniae* (uncured farmers) as opposed to *K. pneumoniae* alone (cured farmers).

3.5 Prudent harvesting: spore counts

In the same experiment, we investigated the effect of removing host-associated bacteria from farmers on the number of spores produced after completion of the social stage. We collected and counted the number of spores produced by cured and uncured farmers and non-farmers after measuring for colony expansion above. We tested whether spore production varies by farmer status and/or curing status by performing a two-way analysis of variance. We found no significant effect of farmer status, found a strongly significant curing effect, and strongly significant interaction between farmer and curing status (Mixed model analysis of variance: Farmer $F_{1,16} = 0.19$ $p = 0.6720$; Cured $F_{1,16} = 16.69$ $p = 0.0009$; Farmer*Cured $F_{1,16} = 17.42$ $p = 0.0007$). Cured farmers produce significantly more spores than uncured farmers and cured and uncured non-farmers (Fig. 5c). Here the difference between uncured farmers and non-farmers is not significant, so in one sense it is meaningless to ask whether the difference is host controlled or symbiont controlled. However the direction (fewer spores in farmers) is the same as in previous experiments with abundant food where the difference was significant (Brock et al. 2011; DiSalvo et al. 2015). But again, the cured farmers are not like either farmers or non-farmers, this time being more extreme than the higher (non-farmer) value. This suggests a complex interaction. Both farmer treatments allow greater expansion of the bacteria, creating a potential advantage over

non-farmers (Fig. 5b), but perhaps the uncured farmers suffer from carriage of *Burkholderia*, while the cured ones do not.

3.6 Resistance to farmer-associated *Burkholderia* Clade 2 trait

We have previously reported that non-farmer spore production is greatly harmed when grown in small amounts of *Burkholderia* Clade 2 bacteria mixed with a food bacterium compared to *Burkholderia* Clade 2 farmers (Brock et al. 2013). This suggests that this trait is caused by differences between farmers and non-farmers but a more complete test requires testing whether this effect also occurs in cured farmers. Here we used five cured farmer and five cured non-farmer *D. discoideum* clones grown in a fixed initial plating amount of 5 % *Burkholderia* Clade 2 and 95 % *K. pneumoniae* compared to 100 % *K. pneumoniae* alone as a control to answer this question. We tested if *Burkholderia* Clade 2 bacteria resistance varies by farmer status and/or bacteria type by performing a two way analysis of variance with replication. We found a strongly significant interaction of bacteria type, and while there were some differences between the two replicates, these did not reach statistical significance (Mixed model analysis of variance: Farmer $F_{1,24} = 0.42$ $p = 0.5371$; Bacteria $F_{1,24} = 68.72$ $p < 0.0001$; Farmer*Bacteria $F_{1,24} = 3.15$ $p = 0.0884$; Replicate $F_{1,24} = 2.94$ $p = 0.0991$; Farmer*Replicate $F_{1,24} = 1.28$ $p = 0.2696$; Bacteria*Replicate $F_{1,24} = 0.83$ $p = 0.3703$; Farmer*Bacteria*Replicate $F_{1,24} = 3.22$ $p = 0.0856$). Spore production on food bacteria alone is the same for cured farmers and non-farmers and both produce fewer spores when grown on 5 % *Burkholderia* Clade 2 (Fig. 6). However cured farmers grown with *Burkholderia* Clade 2 produced significantly more spores than cured non-farmers. Thus, non-farmers are harmed more, confirming there are host-caused differences in this trait. These data suggest *Burkholderia* Clade 2 farmers have evolved resistance to *Burkholderia* Clade 2 bacteria, and this may be an indication of a long association between farmer-associated bacteria and the farmer amoebae.

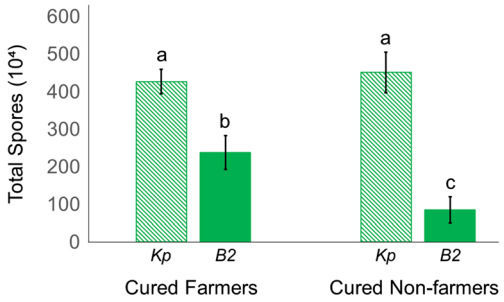

Fig. 6 Cured farmers maintain resistance to farmer-associated *Burkholderia* Clade 2 bacteria and represent a farmer trait. We counted the number of spores from both uncured and cured farmers and non-farmers grown on *Klebsiella pneumoniae* (*Kp*) and *Burkholderia* Clade 2 (*B2*). Cured farmers produced significantly more spores when grown in *Burkholderia Clade 2* compared to cured non-farmers which suggests that farmers have adapted to the farmer-associated bacteria. We used a post hoc Tukey HSD test to correct for multiple comparisons and significant differences are indicated by different letters. Error bars equal s.e.m.; N equals 10

4 Discussion

In this study we explored the extended phenotypic effects in the farming symbiosis between *Dictyostelium discoideum* amoebas and certain *Burkholderia* bacteria. We did this primarily through removing the bacteria from farmers by antibiotic treatment and asking whether traits of these cured farmers closely resemble those of uncured farmers or those of non-farmers who do not carry bacteria. In intimate symbioses, partners can extend their phenotypic effects outside their own bodies and into the bodies and traits of their partners. These effects are presumably usually adaptive to the actor but may be either adaptive or maladaptive to the partner who is affected. Disentangling these effects is easiest when the partnership is facultative, such that one partner can be removed, as was the case in this report.

Here we tested four phenotypic traits (slug migration distance, ability to transport bacteria, prudent harvesting, and resistance to *Burkholderia* Clade 2 bacteria). These are traits associated with the farming symbiosis in *D. discoideum* which we examined to see if they were bacterially conferred by *Burkholderia* Clade 2 symbionts or if they are instead a result of differences between farmers and non-farmers (Table 1).

We found that migration distance is a bacterially conferred trait (Fig. 3). This trait may be maladaptive for the farmer as the farmer-associated bacteria seem to be inhibiting the movement of the farmer slugs. An alternative possibility is that farmer *Dictyostelium* need not disperse as far given that they are carrying food. In general it may be easier to find necessary nutrients for transported bacteria than to locate edible food bacteria as we have previously shown (Brock et al. 2011).

Our lab has recently reported that farmer-associated *Burkholderia* is the leading initiator of stable bacterial carriage in naïve non-farmer *D. discoideum* (DiSalvo et al. 2015). The data presented here extend this result. Both under conditions that suppress slug migration and conditions that encourage slug migration, cured farmers resembled non-farmers in that they rarely carried food bacteria (Fig. 4). These data support the hypothesis that the ability to transport bacteria is a trait that is conferred by the *Burkholderia* symbiont.

The experiment also revealed another interesting aspect of carriage. In the no-migration treatment, even *Dictyostelium* without *Burkholderia* (non-farmers and cured farmers) occasionally carried the food bacterium, but this declined in the corresponding migration treatments though was not significant. These results are consistent with previous research. Raper (1937) reported that spores taken from fruiting

Table 1 Traits associated with the farming symbiosis are found to be bacterially conferred, partially bacterially conferred, or not bacterially conferred

Trait differences between farmers and non-farmers	Conferred by bacteria?
Migration distance	Yes
Bacteria transport with and without migration	Yes
Spore production on farmer-associated *Burkholderia* Clade 2	No
Prudent harvesting: bacteria consumed	Complex
Prudent harvesting: territory expansion	Complex
Prudent harvesting: spore production	Complex

body sori formed at least 0.5 cm outside the area of bacteria colonies would be free from bacteria (in essence sterile) while spores taken from fruiting body sori located within bacteria colonies would still contain bacteria in about one of 10 sori tested. This suggests that non-farmers may occasionally pick up bacteria haphazardly when they fruit in areas with bacteria, but not when they are in areas free of bacteria. This may involve a different mechanism than the consistent internal carriage of farmers (DiSalvo et al. 2015).

Not all traits examined here were bacterially conferred. We have presented data showing that farmer amoebae are more resistant to harm from *Burkholderia* symbionts than non-farmers. This suggests that farmers may have co-evolved with their *Burkholderia* Clade 2 symbionts. We have also previously shown that supernatants from farmer-associated *Burkholderia* Clade 2 isolates harm non-farmers and benefit farmers in the solitary, vegetative stage as well as the social stage (Brock et al. 2013). We found a similar effect from small molecules secreted by a farmer-associated symbiont similar to *Pseudomonas fluorescens* (Stallforth et al. 2013). Interestingly, this farmer associated with two *P. fluorescens* symbionts one of which appears to have evolved edibility in association with the farmer. Based on these data, we propose that resistance to harm from *Burkholderia* Clade 2 is a trait caused by evolved changes in the farmer amoebae and suggests a longer association between the farmer-associated bacteria and the farmer. Alternatively, though less likely, there might simply be unselected variation in *D. discoideum*, which makes some resistant to *Burkholderia* and thus candidates for farming and others non-resistant, so that they die when infected and are collected only as uninfected non-farmers. However, this would seem to imply a stronger difference in the extent of harm than we observe (Fig. 6). This work does not rule out the hypothesis that differences between *D. discoideum* farmers and non-farmers prior to exposure to *Burkholderia* Clade 2 have a role in the differences. Nevertheless, the most parsimonious explanation seems to include evolution of farmers to mitigate the harm of *Burkholderia* that they have been consistently exposed to.

Various aspects of the prudent harvesting trait seem to be have causes more complex than simple host control or simple symbiont control. We have previously reported that farmer clones of *D. discoideum* do not consume all available food bacteria before forming fruiting bodies while non-farmers eat all available bacteria (Brock et al. 2011). This trait could be bacterially controlled if the farmer-associated bacteria were somehow signaling or manipulating the *D. discoideum* farmers to enter the social stage while food bacteria was still available, causing them to maladaptively forego some amount of amoebae proliferation before starvation. Alternatively the trait could be adaptively controlled by farmers, not eating all available food in order to save some for transport. Our data show that the amount of bacteria left uneaten by a cured farmer clone is intermediate (Fig. 5a). It is therefore neither completely controlled by the host-associated bacteria nor completely due to the amoebae but instead a case of mixed control.

More complex results were found for the other two traits with the cured farmers being more extreme than the range defined by uncured farmers and non-farmers. Farmer clones were associated with larger bacterial colonies than non-farmer clones when spotted on a plate, but the cured farmers were the most extreme (Fig. 5b). We do not have a clear explanation for this, but it might result in some way from the bacterial colonies being of different types, usually *K. pneumoniae* (food bacteria) alone, but *K. pneumoniae* (food bacteria) together with carried *Burkholderia* (non-food bacteria) in the uncured farmer treatment. In this same experiment *Dictyostelium* spore production showed the opposite effect in the sense that the cured farmers were more extreme than the non-farmers (Fig. 5c). This could result from a combination of beneficial effects of prudent harvesting by both uncured and cured farmers, coupled with a cost of carrying *Burkholderia* experienced only by the uncured farmers.

Phenotypic control of host traits is not unique to this system. Many symbionts confer phenotypic traits that are no longer expressed (and therefore detectable) if the symbiont is removed. An extreme example was recently reported in an agricultural pest (Himler et al. 2011). Whiteflies infected with *Rickettsia* had a higher survival rate, developed to adulthood faster, and produced more offspring with a female-biased sex ratio. The microbiomes of obese and lean mice can also dramatically affect behavior (Turnbaugh et al. 2006). The phenotype of germ-free mice is transformed to obesity with the

transplant of obese mouse gut microbiota. In another example, an aphid infected with the endosymbiont *Rickettsia* can change body color from red to green (Tsuchida et al. 2010) protecting them from predation from ladybird beetles (Losey et al. 1997). An important future challenge for the *Dictyostelium-Burkholderia* farming system is to further define which traits are advantageous to both parties and which represent detrimental manipulations of one party by the other.

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