Symbiont-Induced Phagosome Changes Rather than Extracellular Discrimination Contribute to the Formation of Social Amoeba Farming Symbiosis

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ABSTRACT Symbiont recognition is essential in many symbiotic relationships, especially for horizontally transferred symbionts. Therefore, how to find the right partner is a crucial challenge in these symbiotic relationships. Previous studies have demonstrated that both animals and plants have evolved various mechanisms to recognize their symbionts. However, studies about the mechanistic basis of establishing protist-bacterium symbioses are scarce. This study investigated this question using a social amoeba Dictyostelium discoideum and their Burkholderia symbionts. We found no evidence that D. discoideum hosts could distinguish different Burkholderia extracellularly in chemotaxis assays. Instead, symbiont-induced phagosome biogenesis contributed to the formation of social amoeba symbiosis, and D. discoideum hosts have a higher phagosome pH when carrying symbiotic Burkholderia than nonsymbiotic Burkholderia. In conclusion, the establishment of social amoeba symbiosis is not linked with extracellular discrimination but related to symbiont-induced phagosome biogenesis, which provides new insights into the mechanisms of endosymbiosis formation between protists and their symbionts.

IMPORTANCE Protists are single-celled, extremely diverse eukaryotic microbes. Like animals and plants, they live with bacterial symbionts and have complex relationships. In protist-bacterium symbiosis, while some symbionts are strictly vertically transmitted, others need to reestablish and acquire symbionts from the environment frequently. Therefore, how to find the right partner is a crucial challenge in their relationship. It has been demonstrated that both animals and plants, such as the legume and squid symbioses, have evolved various mechanisms to recognize their symbionts (7, 8). However, it is unclear whether unicellular protist hosts could recognize and discriminate their symbionts.

KEYWORDS symbiosis, chemotaxis, phagosome, discrimination, Dictyostelium discoideum, Burkholderia

Host-symbiont mutualisms are prevalent in nature and can significantly impact each other’s fitness (1–5). While some symbionts are vertically transmitted, other symbiotic relationships need to reestablish in every generation and acquire symbionts from the environment (6). Therefore, how to find the right partner is a crucial challenge in their relationship. It has been demonstrated that both animals and plants, such as the legume and squid symbioses, have evolved various mechanisms to recognize their symbionts (7, 8). However, it is unclear whether unicellular protist hosts could recognize and discriminate their symbionts.

Protists are unicellular eukaryotic organisms that are not animals, plants, or fungi, which have complex relationships with bacteria, ranging from predation to symbiosis (1, 9–11). For instance, a large number of diverse symbionts can be found in both

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ciliates (12–17) and amoebas (1, 18). Because protists are difficult to culture, and most of their symbionts are unculturable bacteria, our understanding is restricted to a few systems (1, 13, 19–24), and we know very little about the partner choice in protist-bacteria interactions (25). Therefore, we need simple systems in which both partners can be manipulated empirically.

The amoeba proto-farming symbiosis is a promising system to address whether unicellular protist hosts could recognize and discriminate their symbionts (19–21, 25–28). Dictyostelium discoideum is a soil-dwelling amoeba belonging to protozoa and primarily feeding on bacteria, which has been widely used as an ideal system to study cell biology, symbiosis, evolution, and ecology (20, 21, 23, 24, 26, 28–32). Amoebas can aggregate and differentiate into pluricellular fruiting bodies upon food-deprived conditions. Approximately 20% of cells sacrifice to generate stalk, and the remaining cells differentiate into mature spores, resulting in a sorus at the top of the stalk (21). Burkholderia agricolaris and B. hayleyella, two symbiotic bacteria, can form a stable association with D. discoideum hosts. They could not support amoeba growth alone, but they benefit the amoebas by inducing additional bacterial carriage, which can be used to seed new food populations (20, 21). Both symbionts can live on their own, indicating they are facultative symbionts, which raises the question of how the association between D. discoideum and its carried Burkholderia is formed and maintained.

Dictyostelium discoideum is a prime organism to study host-bacterium interactions (33). Our previous study showed that Burkholderia symbionts used chemotaxis to find their amoeba hosts (25). However, it is unclear whether D. discoideum hosts could recognize and discriminate their Burkholderia symbionts. Amoebas interact with bacteria through two steps. First, they use chemotaxis to search and track bacteria. Chemotaxis is the movement of cells toward a chemical gradient, which has significant roles in many biological processes (34). It has been reported that amoebas are attracted to Gram-negative bacteria in a chemotaxis assay (35), but it is not clear whether D. discoideum hosts are more attracted to their Burkholderia symbionts. Second, amoebas use phagocytosis to ingest and feed on bacteria as phagocytes. After engulfment, the phagosomes of amoebas play essential roles in killing and digesting bacteria with the help of acidification, proteases, hydrolases, and ROS (36–39). Previous studies in other systems have shown that the evasion of the lysosomal fusion of the phagosome is mainly due to bacterial mechanisms. It was reported that some components such as ankyrin proteins and MavE effector of L. pneumophila have effects on the interaction with hosts via phagosome biogenesis and lysosomal evasion (40, 41). Bacterial surface traits, including alkaline substances, can partially inhibit the digestion of Tetrahymena pyriformis from enhancing escape rates (42). Some pathogenic bacteria can often survive from phagosome acidification and exist in amoebas by inhibiting phagosome maturation or escaping from phagosomes (18, 43), whereas most bacteria cannot survive within amoebas. Therefore, the bacterium-induced phagosome changes may also contribute to the formation of social amoeba farming symbiosis.

Currently, it is unclear why some bacteria can form symbiotic relationships with D. discoideum hosts while others cannot. In addition, the debate also exists whether hosts can discern symbiotic, nonsymbiotic bacteria, or food bacteria to stabilize relationships with symbiotic bacteria. It may not be accidental that hosts can selectively discriminate bacteria, forming symbiotic relationships in the environment. Therefore, we hypothesize that D. discoideum hosts can distinguish and recognize their Burkholderia symbionts extracellularly (chemotaxis) and intracellularly (phagocytosis). We conducted chemotaxis assays and flow cytometry measurements on phagosome pH to answer the following question: can amoeba hosts discriminate their bacterial symbionts extracellularly or intracellularly?

**RESULTS**

*Dictyostelium discoideum* moved to all bacteria in a one-way chemotaxis assay, but the chemotactic responses were similar. We conducted one-way chemotaxis assays to investigate how *D. discoideum* host responded to food source bacteria *K. pneumoniae*,
symbiotic and nonsymbiotic Burkholderia species (Fig. 1). The numbers of migrated amoebas gradually increased in all treatments with time (2, 4, 6, and 8 h; Fig. 2). By one-way analysis of variance (ANOVA) using Tukey's multiple-comparison test, no significant difference in migrating amoebas responding to all the bacteria was observed at the beginning of 2 h. However, compared to the control group, D. discoideum showed stronger chemotaxis to all targeted bacteria than blank control after 6 h (Fig. 2A to C).

Within carried Burkholderia symbionts, our results showed that their chemotactic responses were similar, and no significant variation was observed within each symbiotic Burkholderia species at 8 h (P > 0.05) (Fig. 2A and B). However, nonsymbiotic Burkholderia species induced different chemotactic responses, in which fewer amoebas were attracted to B. xenovorans than to other B. silvatlantica (P < 0.0001) and B. tuberum (P = 0.02) after 6 h (Fig. 2C).

Overall, amoebas showed positive chemotactic responses to symbiotic B. agricolaris (P = 0.0007), B. hayleyella (P = 0.0048), non-symbiotic Burkholderia (P = 0.004), and the food source K. pneumoniae (P = 0.0002) (Fig. 2D). However, there was no significant difference in amoebas migrating toward the symbiotic B. agricolaris and B. hayleyella compared to nonsymbiotic species (P > 0.05) (Fig. 2D).

D. discoideum could not discriminate different bacteria in a paired choice assay. Furthermore, we performed paired choice assays to investigate how D. discoideum host responded to different bacteria. We separately compared food bacterium K. pneumonia with the other 10 individual bacteria in a paired choice assay and analyzed with a two-tailed Student t test. Our data showed that the number of migrated cells toward bacteria is similar between feeding bacteria and each symbiotic or nonsymbiotic Burkholderia species, suggesting that amoebas show no significant difference of chemotaxis between Klebsiella pneumoniae and most bacterial species (P > 0.05) except for B2qs21 (P = 0.0481) (Fig. 3).

We next compared each nonsymbiotic Burkholderia (including B. unamae, B. silvatlantica, and B. tuberum) to the other six symbiotic Burkholderia for the paired-choice assay. The results showed that only one combination (B. unamae versus B. agricolaris B2qs11) had a significant difference in Dictyostelium migration (P = 0.0377) (Fig. 4), while no significant difference was observed in all other comparisons. These results suggest that D. discoideum host cannot discriminate symbiotic and nonsymbiotic Burkholderia species extracellularly.
Burkholderia bacteria change the phagosome pH of D. discoideum host. Since we found that D. discoideum cannot discriminate its symbionts bacteria extracellularly, next, we investigated whether it could discriminate them intracellularly. Using pH-sensitive fluorescent probes, we measured the phagosome pH by flow cytometry (see Table S1 in the supplemental material). By one-way ANOVA Tukey’s multiple-comparison test, we observed that the phagosome pH of D. discoideum cells infected with Burkholderia was significantly different compared to food bacterium K. pneumoniae except for B. silvatlantica and B. tuberum (P > 0.05) (Fig. 5A).

In addition, nonsymbiotic Burkholderia induced the lowest phagosome pH compared to symbiotic B. agricolaris (P = 0.0008) and B. hayleyella (P < 0.0001) (Fig. 5B). Furthermore, we also observed differences between two symbiotic Burkholderia: B. hayleyella induced a higher phagosome pH than did B. agricolaris (P = 0.0132) (Fig. 5B). These results indicated that symbiotic Burkholderia could inhibit phagosome acidification of D. discoideum host.

DISCUSSION

The symbiotic associations between protists and their symbionts provide an excellent system to study symbiosis because we could culture, mix, and match both partners to test different research questions in ecology and evolution (1, 22). Using D. discoideum as a host...
system, this study showed that symbiont-induced phagosome changes rather than extracellular discrimination contributed to the formation of social amoeba farming symbiosis. We found no evidence that *D. discoideum* could distinguish different *Burkholderia* extracellularly in chemotaxis assays. Instead, symbiont-induced phagosome biogenesis contributed to the formation of social amoeba symbiosis, and *D. discoideum* hosts exhibited higher phagosome pH when carrying symbiotic *Burkholderia* than nonsymbiotic *Burkholderia*.

We found no evidence that amoeba hosts could recognize their symbionts extracellularly. Previous studies have shown that amoebas locate and search prey effectively depending on chemotaxis. It was reported that soluble compounds of secondary metabolites produced by bacteria mediate interactions between *Dictyostelium* and bacteria (44, 45). Consequently, *D. discoideum* has an instinctive response to feed on bacteria or acquire symbionts from the complex environment. Recent studies indicate an intense preference for Gram-negative compared to Gram-positive bacteria (30). However, our study showed that all tested *Burkholderia* bacteria could attract amoebas, but the cells could not distinguish symbiotic and nonsymbiotic *Burkholderia* bacteria. We also found that *D. discoideum*
cannot distinguish K. pneumoniae paired against other Burkholderia, although D. discoideum showed a more robust response to K. pneumoniae in some cases.

Our results support the hypothesis that amoebas may not discriminate different Burkholderia species extracellularly. In addition, the results show that symbiont-induced phagosome biogenesis contributes to the formation of social amoeba symbiosis. Bacteria have complex relationships with amoebas, evolving complex intracellular lifestyles (1). For example, amoebas play roles such as bacterial predators, symbiotic partners, bacterial vehicles or “Trojan horses” and “biological reservoirs” (46). Recent studies have reported that some microorganisms can resist killing by free-living amoebas (47). Some pathogenic bacteria can resist digestion and escape from amoebas to avoid damage from the phagosome, reproduce within the environment, and exploit host resources (48). Our results suggested that specific mechanisms of the symbiotic Burkholderia are to change the pH of the phagosome. As a result, intracellular pathogens would affect phagosome-lysosome fusion (48), which may explain how the symbiotic Burkholderia can survive in the phagosome and form a stable symbiotic relationship with the host Dictyostelium. Interestingly, although the nonsymbiotic Burkholderia species have similar edibility to the amoeba host (49), one species B. xenovorans, induced a higher phagosome pH than B. unamae, B. silvatlantica, and B. tuberum. In addition, phylogenetically, B. xenovorans is also closer to the symbiotic Burkholderia, indicating a possible correlation between phylogeny and phagosome acidification disruption.

This study also provides new insights into the relationships between Dictyostelium and Burkholderia and suggests a potential mechanism of bacterial food carrying. Symbiotic Burkholderia bacteria have a similar survival strategy with pathogens, and previous studies also showed that harboring Burkholderia imposed fitness costs on Dictyostelium hosts (19, 20). Therefore, we believe that the Dictyostelium-Burkholderia symbiosis is or has evolved from a more parasitic relationship. In addition, the induction of bacterial food carrying is likely the result of symbiont-induced phagosome changes. Only symbiotic Burkholderia can form a stable relationship with amoeba and induced bacterial carriage instead of nonsymbiotic Burkholderia. This symbiotic specificity mechanism occurs due to pH variations in the intracellular environment. Therefore, a higher phagosome pH plays a crucial role in maintaining Dictyostelium-Burkholderia symbiosis, which in turn creates a moderate host niche that allows other food bacteria to survive. Future research should focus on the precise molecular mechanisms of the inhibition of phagosome acidification in these symbiotic Burkholderia bacteria.

FIG 5 Phagosome pH of amoebas in response to different bacteria. The same experiment was plotted separately by bacterium (A) and group (B). (A) Comparison among different strains. Compared to other different clones, the pH was significantly changed in amoebas with K. pneumonia (n = 3; the error bar represents the SD). (B) Comparison among symbiotic and nonsymbiotic bacteria. For panels A and B, the dark symbols indicate the phagosome pH of amoebas responding to K. pneumonia. The light orange and the deep orange symbols indicate the phagosome pH of amoebas responding to symbiotic B. agricolaris and B. hayleyella, respectively. The gray symbols indicate the phagosome pH of amoebas responding to the nonsymbiotic bacteria B. unamae, B. silvatlantica, and B. tuberum. For panels A and B, asterisks indicate significance (*, P < 0.05; **, P < 0.001; ****, P < 0.0001) according to the one-way ANOVA Tukey’s multiple-comparison test.
TABLE 1  Bacterial strains used in this study

| Bacterial strain | Type            | Gram stain       | Reference |
|------------------|-----------------|------------------|-----------|
| B. agricolaris   | Symbiotic       | Gram negative    | 21        |
| B1qs70           |                 |                  |           |
| B1qs159          |                 |                  |           |
| B1nc21           |                 |                  |           |
| B. hayleyella    | Symbiotic       | Gram negative    | 21        |
| B2qs11           |                 |                  |           |
| B2qs21           |                 |                  |           |
| B2nc28           |                 |                  |           |
| B. unamae        | Nonsymbiotic    | Gram negative    | 25        |
| B. tuberum       | Nonsymbiotic    | Gram negative    | 25        |
| B. silvatlantica | Nonsymbiotic    | Gram negative    | 25        |
| B. xenovorans    | Nonsymbiotic    | Gram negative    | 25        |
| K. pneumoniae    | Nonsymbiotic    | Gram negative    | 21        |

MATERIALS AND METHODS

Dictyostelium strains and culture conditions. Wild D. discoideum clones QS9 was used in this study (21). Frozen D. discoideum spores were grown on SM/5 agar plates (2 g glucose, 2 g Bacto peptone [Oxoid], 2 g yeast extract [Oxoid], 0.2 g MgCl2, 1.9 g KH2PO4, 1 g K2HPO4 and 15 g agar per L), mixed with the food bacterium K. pneumoniae, and cultured in a light incubator at 21°C.

Bacterial strains and culture conditions. K. pneumoniae and 10 Burkholderia isolates, including 6 carried Burkholderia and 4 noncarried Burkholderia described in previous work (20, 50), were used in this study. The symbiotic Burkholderia contains B. agricolaris (B1qs70, B1qs159, and B1nc21) and B. hayleyella (B2qs11, B2qs21, and B2nc28). The bacteria from the frozen clonal isolate were incubated on SM/5 agar medium for approximately 2 to 5 days at 21°C. Bacterial strain information is presented in Table 1.

Extracellular discrimination: chemotaxis assay. Based on the 16S rRNA gene phylogeny (20, 25), two distinct clades, including symbiotic B. agricolaris and B. hayleyella, were selected for chemotaxis assays together with four nonsymbiotic Burkholderia bacteria (Fig. 1). To detect the diverse chemotactic responses of D. discoideum to different bacteria, the methods were shown as follows. 2 × 10⁷ spores were suspended with 200 μL of K. pneumoniae (optical density at 600 nm [OD600] = 1.5) in starvation buffer (2.2 g KH2PO4 and 0.7 g K2HPO4 per L). Amoeba log growth occurs about 36 h after plating spores (28). At this time, log-growth amoebas were collected in the starvation buffer from the petri dishes for the chemotaxis experiment and centrifuged the collected amoebas/bacterial suspension at 1,500 x g for 3 min to wash the amoebas clean from the bacteria.

The pelleted amoebas were washed in an excess volume of ice-cold starvation buffer three or four times to get rid of residual bacteria. Each bacterial suspension in starvation buffer was prepared at an OD600 of ~1.5. Furthermore, we tested one-way chemotaxis and paired-choice assay, respectively. 2 μL of amoeba suspension was spotted on 2% Noble agar, and 2 μL of each bacterial suspension was spotted onto one side or two different bacterial pair on both sides, which was measured at a 0.65-cm distance from the amoeba suspension. A grid was placed beneath the plate to ensure equal distances. After being spotted at room temperature, the number of migrated amoebas that moved toward bacteria was counted at different time points (2, 4, 6, and 8 h). We used a microscope with 20 x lens objective (200 x total magnification) in the bright field to manually count the migrated amoeba numbers (Fig. 1B). All tests were done in three biological replicates.

Measurement of phagosome pH using flow cytometry. Log-growth amoebas infected with different Burkholderia samples were used in this study. To set up the experiment, we mixed the specified Burkholderia (OD600 = 1.5) at 5% (10 μL) and K. pneumoniae at a 95% (190 μL) volume and plated D. discoideum spores (2 × 10⁹) with 200 μL of the bacterial mixture on SM/5 plates in a light incubator at 21°C. The amoeba cells at the exponential stage were collected after 36 h. The amoeba suspension was collected and rinsed three times at 1,500 × g for 3 min to remove the remaining bacteria. Cells were incubated with dextran coupled to Oregon green (250 μg/mL; Invitrogen), a pH-sensitive probe combined, and a pH-insensitive probe Alexa 647 (30 μg/mL; Invitrogen) to label amoebas that carried symbiotic Burkholderia or nonsymbiotic Burkholderia (51). After 20 min, the cells were pelleted (1,500 × g, 3 min) and rinsed once for further flow cytometer analyses. All tests were done in three biological replicates.

A flow cytometer (Accuri C6 cytometer; BD, USA) was used to measure endosomal pH in cells. The FL1 channel was applied to measure the Oregon green fluorescence with an excitation wavelength of 488 nm and an emission of 515 to 545 nm, while the FL4 channel was used to measure Alexa 647 fluorescence with an excitation of 632 nm and an emission of 655 to 695 nm. At least 10,000 cells were detected, with the median fluorescence values (see Table S1 in the supplemental material). The background autofluorescence values were subtracted from cells without exposure to fluorescent dyes. A calibration curve was prepared in each experiment. After being mixed with fluorescent dextran for 20 min, the cells were washed and resuspended in ice-cold HLS at the indicated pH values (pH 3, 4, 5, 6, 7, and
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