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Letter

Facultative symbiont virulence determines horizontal transmission rate without host specificity in *Dictyostelium discoideum* social amoebas

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Abstract

In facultative symbioses, only a fraction of hosts are associated with symbionts. Specific host and symbiont pairings may be the result of host–symbiont coevolution driven by reciprocal selection or priority effects pertaining to which potential symbiont is associated with a host first. Distinguishing between these possibilities is important for understanding the evolutionary forces that affect facultative symbioses. We used the social amoeba, *Dictyostelium discoideum*, and its symbiont, *Paraburkholderia bonniea*, to determine whether ongoing coevolution affects which host–symbiont strain pairs naturally cooccur within a facultative symbiosis. Relative to other *Paraburkholderia*, including another symbiont of *D. discoideum*, *P. bonniea* features a reduced genome size that indicates a significant history of coevolution with its host. We hypothesized that ongoing host–symbiont coevolution would lead to higher fitness for naturally cooccurring (native) host and symbiont pairings compared to novel pairings. We show for the first time that *P. bonniea* symbionts can horizontally transmit to new amoeba hosts when hosts aggregate together during the social stage of their life cycle. Here we find evidence for a virulence–transmission trade-off without host specificity. Although symbiont strains were significantly variable in virulence and horizontal transmission rate, hosts and symbionts responded similarly to associations in native and novel pairings. We go on to identify candidate virulence factors in the genomes of *P. bonniea* strains that may contribute to variation in virulence. We conclude that ongoing coevolution is unlikely for *D. discoideum* and *P. bonniea*. The system instead appears to represent a stable facultative symbiosis in which naturally cooccurring *P. bonniea* host and symbiont pairings are the result of priority effects.

Keywords: coevolution, facultative symbiosis, flow cytometry, horizontal transmission, protist, virulence factor

Lay summary

Symbiotic relationships between hosts and their microbial partners are prolonged and intimate associations. Some of these relationships are obligatory for both a host and symbiont to survive, while others are facultative and each partner can survive without the other. In the latter case, some host individuals may be associated with a symbiont while others are not. Specific host and symbiont pairings can be the result of reciprocal adaptation between host and symbiont partners so that naturally cooccurring pairings are best suited for each other in terms of their biological fitness. On the other hand, the symbiont that a host is associated with may simply be the symbiont that arrived first, in what is called a priority effect. We sought to determine which possibility best explained naturally cooccurring pairings of host and symbiont strains of the social amoeba *Dictyostelium discoideum* and its symbiont *Paraburkholderia bonniea*. Our work demonstrates that *D. discoideum* and *P. bonniea* are in a stable facultative relationship. Specific host and symbiont pairings appear to be the result of priority effects, and *D. discoideum* hosts without symbionts are simply uncolonized. This work fills a gap in our understanding of the evolutionary forces affecting facultative symbiotic relationships. We also show for the first time that *P. bonniea* symbionts can spread to new amoeba hosts when hosts aggregate together during the social stage of their life cycle.

Introduction

Host–symbiont associations range from obligate to facultative based on the degree of host–symbiont dependency (Fisher et al., 2017; Sachs et al., 2011). For obligate symbioses in which host and symbiont need each other to survive, we expect significant coadaptation to have occurred between hosts and symbionts often over a longer period of coevolution (Law & Dieckmann, 1998). In contrast, for facultative symbioses in which host and symbiont can each survive in a free-living state, we expect a lesser degree of coadaptation or relationships to be more recent (Lo et al., 2016). Significant coadaptation in obligate symbioses, especially those that feature strict vertical transmission of symbionts from parent to offspring, often results in symbionts with highly reduced genome sizes (McCutcheon & Moran, 2012; Moran et al., 2008). In facultative symbioses, many of which include horizontal modes of symbiont transmission from nearby hosts or the environment,

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symbiont genome sizes can vary widely but are on average intermediate in size (Fisher et al., 2017; Lo et al., 2016; Sachs et al., 2011; Toft & Andersson, 2010). While ongoing coevolution can lead to continued genome reduction for a facultative symbiont, genome size may be stable if the facultative symbiosis is evolutionarily stable. For example, nitrogen-fixing facultative symbionts in the genus *Frankia* include *Frankia alni* strain ACN14a, which appears to be a facultative symbiont with a stable genome size and evolutionarily stable relationship with its hosts. In contrast, *Frankia* sp. (cluster 3) strain EAN1pec and *Frankia* sp. (cluster 1) strain HFPCcl3 have, respectively, evolved reduced and expanded genome sizes (Normand et al., 2007).

In many facultative symbioses, some but not all hosts are associated with a symbiont. What factors determine association patterns of facultative symbionts among individuals of a host species are poorly understood (Niepoth et al., 2018). Understanding why specific individual host and symbiont strains are associated with each other can inform us of how and why facultative symbioses evolve. Possible explanations behind specific patterns of association include ongoing host–symbiont coevolution driven by reciprocal selection, or priority effects that are fitness-neutral with respect to the host–symbiont interaction itself (Ganesan et al., 2022). Mutualistic coevolution could lead to specific pairings of host–symbiont strains that enhance each other's fitness relative to other pairings (Rafaluk-Mohr et al., 2018). In contrast, priority effects in a symbiosis context pertain to the order in which symbionts colonize a host and are typically considered when multiple symbiont species or strains coexist within the same environment as the host (Ganesan et al., 2022). If we extend priority effects to unassociated individual hosts in a facultative symbiosis, presently unassociated individuals may simply be uncolonized. In this sense, the question of what drives host–symbiont association patterns within a facultative symbiosis is related to questions of how host–microbe associations originate (Sieber et al., 2021) and how microbial communities are assembled (Tucker & Fukami, 2014).

The potential for, and outcome of, long-term association between a host and any potential symbiont is determined by intrinsic factors such as host and symbiont genetics, as well as various extrinsic environmental factors (Schmid-Hempel, 2011). For this work, we focused on the intrinsic factors of host defense and symbiont virulence and their potential contributions to fitness consequences of host–symbiont interactions. Host resistance (the ability to limit the extent of an infection) and tolerance (the ability to tolerate the physiological consequences of an infection) are the two large categories of host defense components that affect evolutionary outcomes for symbioses (Ayres & Schneider, 2012; Råberg et al., 2007; Simms & Triplett, 1994). The equivalent symbiont virulence components are proliferation (the ability to proliferate to a certain extent in an infection) and benevolence (the ability to cause beneficial or harmful physiological consequences during an infection) (Wollein Waldetoft et al., 2020). Given a specific host–symbiont pairing, variable host resistance and symbiont proliferation will lead to variation in symbiont density, while variable host tolerance and symbiont benevolence will lead to variation in host fitness consequences relative to symbiont density (Figure 1). It is important to be able to differentiate among components of host defense or symbiont virulence because of their downstream evolutionary effects. For example, host resistance often limits the spread of infections, while host tolerance can do the opposite and cause infections to increase in frequency in a population (Råberg, 2014; Roy & Kirchner, 2000). Targeting pathogen virulence via benevolence rather than proliferation may be more likely to prevent counter adaptation such as the evolution of antimicrobial resistance (Wollein Waldetoft et al., 2020).

We used the *Dictyostelium discoideum–Paraburkholderia bonniea* facultative symbiosis to determine whether ongoing host–symbiont coevolution affects which host–symbiont strain pairs naturally cooccur. The amoeba *D. discoideum* is an established model for understanding intracellular pathogen infections (Steinert & Heuner, 2005). It forms a facultative symbiosis with three species of *Paraburkholderia*: *P. agricolaris*, *P. bonniea*, and *P. hayleyella* (Brock et al., 2020; DiSalvo et al., 2015). Roughly one quarter of soil-isolated *D. discoideum* strains carry one or occasionally multiple species of these symbiotic *Paraburkholderia* (Haselkorn et al., 2019). The three symbionts also provide an opportunity to contrast the effect of different evolutionary histories of association with *D. discoideum*. The sister species *P. bonniea* and *P. hayleyella* (approximately 4.1 million base pairs) have evolved genomes that are half the size of other *Paraburkholderia*, including *P. agricolaris* (approximately 8.7 million base pairs) in the amoeba host environment (Noh et al., 2022). *Paraburkholderia agricolaris* and *P. hayleyella* were previously examined for evidence of host–symbiont coevolution (Garcia et al., 2019; Shu et al., 2018). Naturally cooccurring (native) *P. hayleyella* hosts had a fitness advantage over novel hosts when cured and reinfected with *P. hayleyella*, while native *P. agricolaris* hosts did not have a similar advantage for *P. agricolaris* infections (Shu et al., 2018). *Paraburkholderia hayleyella* was more abundant in soil microcosms (including within-host cells) when native *D. discoideum* hosts were present than when hosts were absent (Garcia et al., 2019). This pattern was not observed for *P. agricolaris*. Therefore, reciprocal selection appears to be present for native *P. hayleyella* hosts and symbionts.

We hypothesized that ongoing host–symbiont coevolution would lead to higher fitness for native host and symbiont pairings compared to novel pairings. We predicted coadapted intrinsic host and symbiont factors would contribute to fitness differences in host–symbiont associations. Because *D. discoideum* form social groups and symbiont horizontal transmission should be facilitated by these social groups, we examined host and symbiont fitness at the group level to better consider the evolutionary consequences of variation in fitness (Alizon & Michalakis, 2015). We expected to find evidence of ongoing coevolution for *D. discoideum–P. bonniea* based on previous results from *P. hayleyella*, the sister species to *P. bonniea*, that contrasted from *P. agricolaris*.

Methods

Overview of host life cycle and experimental procedure

Dictyostelium discoideum has single-cell and multicell stages in its life cycle, regardless of whether *Paraburkholderia* symbionts are present. Vegetative single-cell amoebas feed on bacteria until food density becomes low and starvation begins. The social stage begins when starving amoebas secrete a cyclic adenosine monophosphate (cyclic-AMP) signal (Loomis, 2014). During the social stage, amoebas move up cyclic-AMP gradients and aggregate into multicellular forms that ultimately become fruiting bodies. Amoebas that survive the social stage become spores contained within the fruiting body sorus (Strassmann & Queller, 2011).

Social stages in the lab can occur when amoebas run out of food bacteria on a petri dish (unmanipulated), or when amoebas are removed from food bacteria (manipulated). We used the

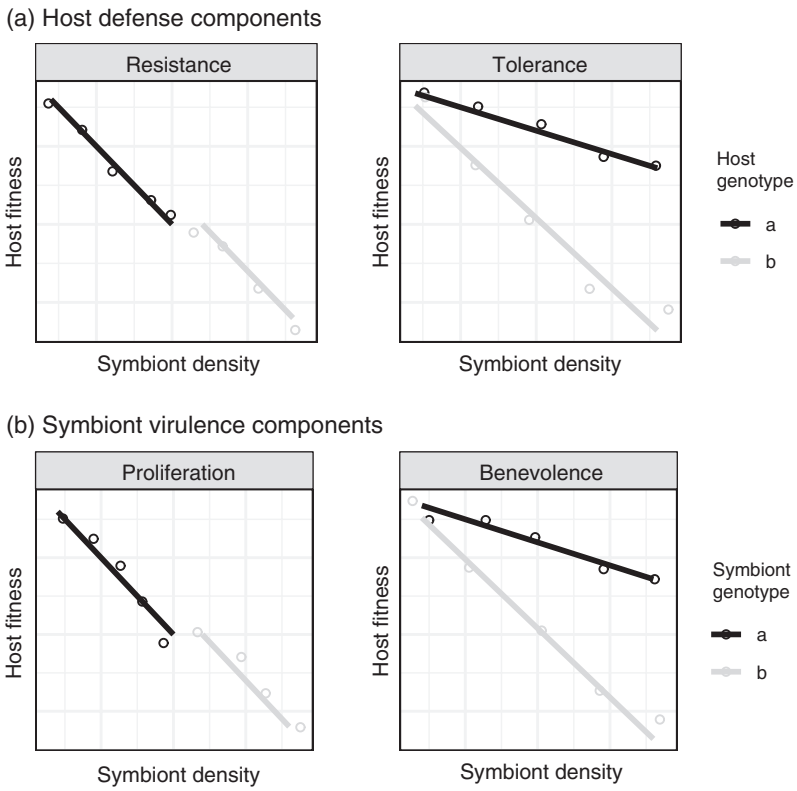

Figure 1. Theoretical differences between resistance-based and tolerance-based host defense, and proliferation-based and benevolence-based symbiont virulence. Both types of variation can be detected by measuring host fitness and detecting when (A) different host genotypes vary in responses to infection by the same symbiont or (B) the same host genotype varies in responses to infection by different symbiont genotypes. (A) Hosts may vary in **resistance** to the same symbiont. More resistant hosts (A—black line; lower average symbiont density) will have overall higher fitness compared to less resistant hosts (B—gray line; higher average symbiont density). But the fitness cost per increase in symbiont density is similar for both hosts, as indicated by the similar slopes. On the other hand, hosts may vary in **tolerance** to the same symbiont. More tolerant hosts (A—black line; shallower slope) may have a similar average symbiont density to less tolerant hosts (B—gray line; steeper slope), but will suffer a lower fitness cost per increase in symbiont density. (B) The same host may suffer different fitness effects due to symbionts that vary in **proliferation**. The better proliferating symbiont (B—gray line; higher average symbiont density) will impart higher fitness costs to the host than the worse proliferating symbiont (A—black line; lower average symbiont density). But the fitness cost to the host per increase in symbiont density is similar for both symbionts. The same host may be infected with symbionts that vary in **benevolence**. Relatively benevolent symbionts (A—black line; shallower slope) will impart a lower fitness cost per increase in symbiont density compared to relatively malevolent symbionts (B—gray line; steeper slope), though both symbionts may reach similar average densities. (Framework adapted from Råberg et al., 2007 and Wollein Waldetoft et al., 2020.)

vegetative stage to expose *D. discoideum* spores to *P. bonniea* symbionts and used spore counts after an unmanipulated social stage as a measure of host fitness (Figure 2A). We then used a subsequent manipulated social stage to expose uninfected amoebas to preinfected amoebas and estimated symbiont horizontal transmission after this social stage as an important aspect of symbiont fitness (Figure 2B).

Focal host and symbiont strains

We used three native host strains of *D. discoideum* (QS395, QS433, QS859) and three novel host strains (QS4, QS17, QS18). The three *P. bonniea* symbiont strains (bb395, bb433, bb859) were each isolated from the native host strains with matching numerical identification codes. All *D. discoideum* strains were previously isolated from Mountain Lake Biological Station in Virginia, USA. The native host strains had been cured of their symbionts using tetracycline and verified as symbiont free using polymerase chain reactions (DiSalvo et al., 2015). The novel host strains were originally isolated without *Paraburkholderia* symbionts and were similarly verified as symbiont free. For each replicate, host (with food bacteria *Klebsiella pneumoniae*) and symbiont strains were grown from freezer stock on SM/5 plates (2 g glucose, 2 g BactoPeptone (Oxoid), 2 g yeast extract (Oxoid), 0.2 g $MgCl_2$, 1.9 g KH_2PO_4, 1 g K_2HPO_4 and 15 g agar per liter). KK2 buffer (2.2 g KH_2PO_4 monobasic and 0.7 g K_2HPO_4 dibasic per liter) was used throughout for handling bacteria, *D. discoideum* spores and amoebas.

Host fitness

We estimated host fitness at a range of infection prevalence for each host–symbiont pairing (6 host strains × 3 symbiont strains). Similar to previous studies (DiSalvo et al., 2015; Garcia et al., 2019; Shu et al., 2018), we estimate host fitness at the social group level rather than at the individual amoeba host level. Instead of symbiont density within an individual host (e.g., Figure 1), we estimate symbiont infection prevalence within a group of amoebas. In contrast to previous studies, we estimate host fitness in the form of group spore production as a function of symbiont infection prevalence, rather than as point estimates given specific MOI.

Experiment

For each pairing, *D. discoideum* spores from freshly grown fruiting bodies were collected and deposited on SM/5 plates at a density of 2×10^5 spores per plate with RFP-labeled symbionts at multiplicities of infection (MOI) of 0 (control), 0.6, 3, and 15. We made triplicate sample plates per pairing and left these plates to fruit at 21 °C for 5–7 days. We then collected all fruiting bodies for each

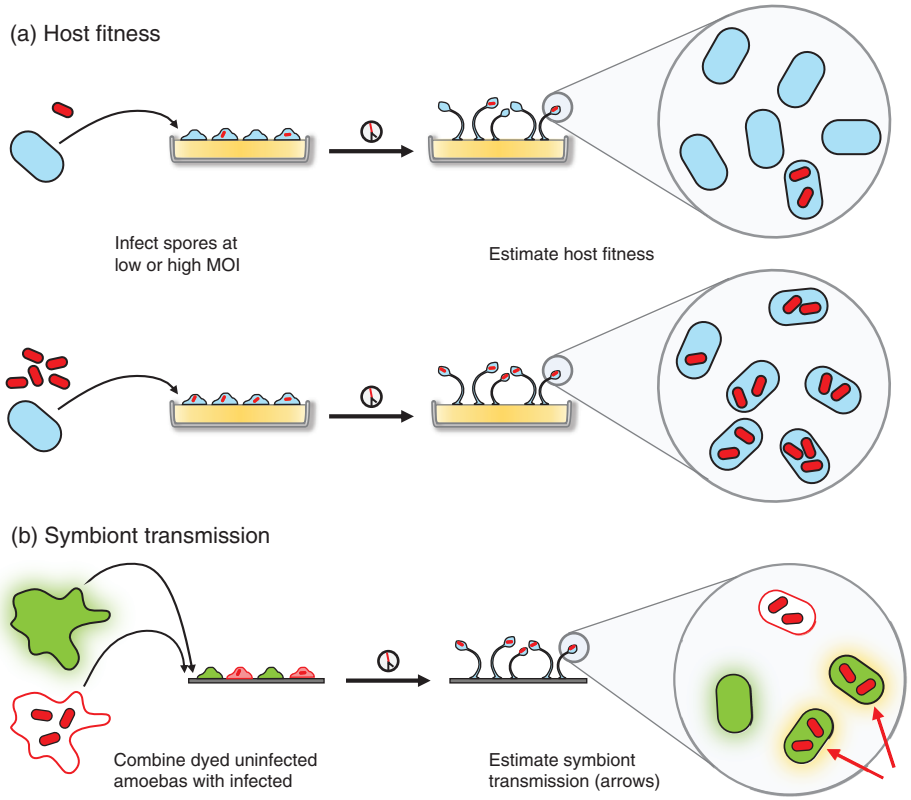

Figure 2. Experimental overview. Vegetative single-cell amoebas feed on bacteria until food density becomes low. Starving amoebas enter the social stage and the amoebas that survive this stage become spores contained within the fruiting body sorus. (A) We exposed *D. discoideum* spores to *P. bonniea* symbionts at multiplicities of infection (MOI) designed to generate a range of infection prevalence in a group of amoeba hosts. We then used spore counts after a vegetative stage–social stage cycle to measure host fitness. (B) We exposed uninfected amoebas to preinfected amoebas and estimated symbiont horizontal transmission after a social stage as an important aspect of symbiont fitness.

sample and estimated the number of spores by counting 50× diluted spores on a hemocytometer and multiplying these counts by the total volume of spores.

Analysis

The MOI range was designed to generate a range of infection prevalence in order to examine the relationship between host fitness and infection prevalence. We estimated infection prevalence (percent of RPF+-infected spores in a sample of 100,000) by running a sample of 100,000 spores from each plate on a BD FACSCalibur flow cytometer (Becton, Dickinson and Company, Franklin Lakes, NJ). FCS files were imported into FlowJo v10.8.1 (*FlowJo Software*, 2023) for analysis, where we applied gates for spores, and then infected spores. For host fitness, we divided total spore counts from each infected sample by the mean spore count of uninfected controls prepared at the same time. This resulted in a relative estimate of spore production at a given level of infection prevalence.

Symbiont transmission

We estimated symbiont horizontal transmission for each host–symbiont pairing (6 host strains × 3 symbiont strains). We estimate symbiont transmission at the social group level rather than at the individual amoeba host level using the social stage of the *D. discoideum* life cycle. For symbiont transmission, we estimate the rate of transmission that occurs within a social group of interacting hosts given a previous degree of symbiont infection prevalence, rather than transmission from one host to another.

Preinfection

Dictyostelium discoideum spores from freshly grown fruiting bodies were collected and deposited on SM/5 plates at a density of 2×10^5 spores per plate with RFP-labeled symbionts at MOI of 0 (control), plus an additional 3–5 MOI ranging from 0.3 to 30. Once these plates had fruited and amoebas were preinfected, we collected fresh spores and deposited them on SM/5 plates at densities of 1×10^5 and 2×10^5 spores per plate with only food bacteria.

Experiment

When amoebas were at log-phase growth roughly 36 h later, uninfected amoebas were dyed with CellTracker Green CMFDA (Invitrogen) dissolved in DMSO. Preinfected amoebas (RFP+) were carried along with the dyed amoebas, only exposed to DMSO, and treated in the same way through room temperature incubation and washes. Afterward, dyed amoebas were combined with infected amoebas at 1:0 (dyed only, negative control), 0:1 (infected only), and 1:1 (mixed) ratios and placed on nitrocellulose filters to aggregate and develop into fruiting bodies. Sample filters were prepared in triplicate. 5–7 days later, we collected all fruiting bodies and their spores from these filters.

Analysis

The MOI range was designed to generate a range of infection prevalence in order to examine the relationship between symbiont transmission and infection prevalence. We estimated infection prevalence from the infected only sample and horizontal transmission from the mixed sample as indicated by the cooccurrence

of dye and infection in a spore. As above, we ran samples of 100,000 spores each on a BD FACSCalibur flow cytometer and imported FCS files into FlowJo v10.8.1 for analysis. We applied gates for spores, then dyed spores from the negative control, and infected spores from infected only samples. A logical (AND) gate was applied to the mixed sample to find the percent of spores that were previously uninfected dyed amoebas that were now positive for symbiont infection (Supplementary Figure S1).

Statistical analysis

Statistical analyses were performed in R v3.6.0 and with packages car v.3.0-12 (Fox & Weisberg, 2019) and lme4 v.1.1-28 (Bates et al., 2015). For both host fitness and symbiont transmission, we fit linear models with mixed effects. For all models, we fit the most complex model first, then removed nonsignificant terms and compared the simpler model with the complex one using the anova() function and its chi-squared test. We examined residuals of the final models for any indications that we had violated model assumptions.

For host fitness, we tested how symbiont strain and host type (native or novel) affected the relationship between infection prevalence and spore production (percent spores produced relative to uninfected controls). We tested the effect of host type by coding the native pairings either strictly (e.g., only QS859 infected with bb859 would be considered native) or leniently (e.g., any native host of *P. bonniea* infected with any *P. bonniea* strain is considered native). We used infection prevalence as a continuous predictor and experiment date and host strain as random effects. For symbiont transmission, we fit a similar model but with transmission rate (percent newly infected spores that were previously uninfected amoebas) as the dependent variable. We examined the effect sizes of factors in the final models using the package effectsize v.0.7.0 (Ben-Shachar et al., 2020) and its epsilon_squared() function. For both host fitness and symbiont transmission, we used the package emmeans v.1.7.2 (Searle et al., 1980) and its emtrends() function for post hoc tests of significant differences between pairwise slopes by symbiont strain. *p*-Values were adjusted using Tukey's method.

We also compared infection prevalence across the two experiments by fitting linear models with host strain as a random effect and the different number of social stages (non-numeric factor; one for host fitness, two for symbiont transmission) and symbiont identity as fixed effects. We used the emmeans() function for post hoc tests of significant differences between means of infection prevalence by symbiont strain. *p*-Values were adjusted using Tukey's method.

Genome analysis

We sequenced and assembled the genomes of bb395 and bb433 as follows: High-molecular-weight DNA was extracted using Lucigen MasterPure Complete DNA and RNA purification kits (LGC, Teddington, UK). Extracted DNA was sent to University of Washington PacBio Sequencing Services for PacBio HiFi sequencing and MiGS for Oxford Nanopore (ONT) and illumina sequencing. Raw reads were cleaned using filtlong v0.2.1 (Wick, 2017/2023) and fastp v0.23.1 (Chen et al., 2018), then assembled and polished using Trycycler v0.5.3 (Wick et al., 2021), and Polypolish v0.5.0 (Wick & Holt, 2022). Trycycler input files were created using Flye v2.9.1-b1780 (Kolmogorov et al., 2019) for both types of reads, Hifiasm v0.18.5-r499 (Cheng et al., 2022) for PacBio reads, and Raven v1.8.1 (Vaser & Šikić, 2021) for ONT reads. Assembled contigs were re-oriented with Circulator v1.5.5 (Hunt et al., 2015). Genes were predicted using Prokka v1.14.6 (Seemann, 2014). Pseudofinder v1.0 (Syberg-Olsen et al., 2022) was used to remove pseudogenes. Pan-genome analysis of the three *P. bonniea* genomes was performed using Roary v3.13.0 (Page et al., 2015). For examination of the differences between genomes, whole genomes were aligned using progressiveMauve (Darling et al., 2010) within Geneious Prime v2020.2.5 (https://www.geneious.com).

Results

Host fitness

We estimated the relationship between host fitness and symbiont infection prevalence by exposing *D. discoideum* hosts to three strains of *P. bonniea* at a range of MOI in native and novel pairings. Native hosts were *D. discoideum* strains harboring their own *P. bonniea* symbiont strain when they were isolated from the wild. Host fitness decreased with increasing infection prevalence across all pairings of hosts and symbionts ($\chi^2 = 376.002$, $p < .001$), but there was no difference between native and novel host–symbiont pairings ($\chi^2 < 0.001$, $p = .981$) (Figure 3; Table 1). This was regardless of whether native status was defined using a strict or lenient definition (Supplementary Table S1). Instead, symbiont strain identity significantly affected how host fitness responded to infection prevalence ($\chi^2 = 23.886$, $p < .001$). For example, bb859 infections resulted in a significantly shallower slope in host fitness decline ($\beta = -0.118$, SE = 0.0172) compared to bb395 ($\beta = -0.180$, SE = 0.0158; bb395–bb859: $p = .025$) and bb433 infections ($\beta = -0.231$, SE = 0.0157; bb433–bb859: $p < .001$) (Figure 3). The effects of bb395 and bb433 infections on host fitness were not significantly different from each other (bb395–bb433: $p = .054$). These results support a significant effect of symbiont benevolence-based virulence variation (Figure 1B) on host fitness during initial infection. Based on our results, bb859 is relatively benevolent, while bb395 and bb433 are relatively malevolent to all hosts.

Symbiont transmission

We estimated the relationship between symbiont transmission and symbiont infection prevalence by exposing uninfected amoebas to preinfected amoebas during a manipulated social stage of the host life cycle in native and novel pairings. Horizontal transmission was detected when previously uninfected spores harbored *P. bonniea* at the end of this social stage. The degree of symbiont horizontal transmission increased with the infection prevalence of preinfected amoebas that entered the social stage ($\chi^2 = 484.965$, $p < .001$) (Figure 4). This positive correlation confirms that the increase in infection is due to horizontal transmission rather than vertical transmission (Ebert, 2013). This is consistent with previous reports that very few amoebas go through cell division during the social stage (Muramoto & Chubb, 2008). As with host fitness above, native and novel host–symbiont pairings were no different in overall patterns of symbiont transmission ($\chi^2 = 2.092$, $p = .148$), regardless of how native status was defined (Table 2; Supplementary Table S2).

In this experiment, infected bb859 reached higher infection prevalence and showed a higher rate of horizontal transmission compared to bb395 and bb433 (Figure 4). Preinfection was established in one social stage, and the experiment was performed in a subsequent social stage. The three *P. bonniea* strains used in this experiment do not significantly differ in their per amoeba-spore bacterial density (Miller et al., 2020). Therefore, higher infection prevalence in bb859 compared to the other strains does not

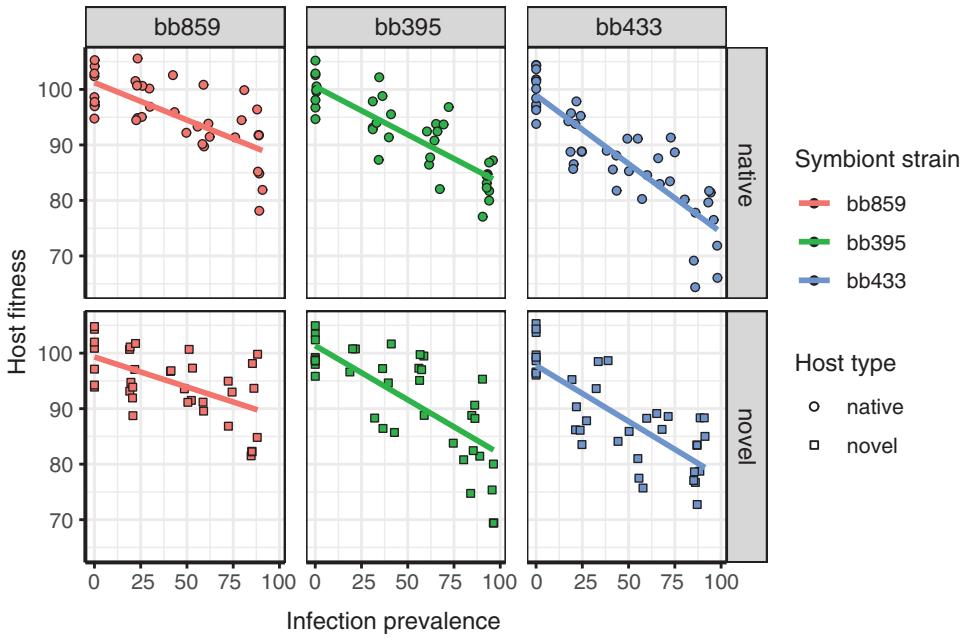

Figure 3. Host fitness was negatively correlated with infection prevalence and differed in slope but not average infection prevalence among symbiont strains after a single social stage. Infection prevalence was estimated per host–symbiont–MOI sample as the percent of infected spores that had RFP-labeled symbionts in them. Host fitness was estimated by the percent of spores produced per host–symbiont–MOI sample, relative to uninfected hosts prepared at the same time as the infected host–symbiont pairings. There was no significant difference between native (top row; lenient definition) vs. novel (bottom row) hosts.

Table 1. Analysis of deviance table for host fitness with lenient native definition

Variable	χ^2	df	*p*-Value	$\eta^2 p$	95% CI
Infection prevalence	376.002	1	<.001	0.63	(0.56, 1)
Symbiont strain	17.998	2	<.001	−0.06	(0, 1)
Host type	<0.001	1	.981	−0.37	(0, 1)
Infection prevalence: Symbiont strain	23.886	2	<.001	0.09	(0.04, 1)

necessarily indicate that bb859 is better at proliferation inside amoeba hosts (Figure 1B). However, bb859 symbiont transmission occurred at a significantly higher rate (β = 0.525, SE = 0.0244) compared to bb395 (β = 0.361, SE = 0.0343; bb859–bb395: $p < .001$) and bb433 (β = 0.249, SE = 0.0289; bb859–bb433: $p < .001$) (Figure 4). The different slopes of transmission rate support a significant effect of symbiont benevolence-based virulence variation (Figure 1B) on symbiont transmission. When native hosts were leniently defined but not when strictly defined, infection prevalence and host type interacted to reveal that novel hosts became infected by bb859 at a slightly higher density compared to native hosts and therefore also transmitted symbionts at a higher level ($\chi^2 = 35.991$, $p < .001$).

Synthesis of host and symbiont fitness

We observed a significant difference in infection prevalence between the two experiments that is likely due to differences in symbiont strain-specific virulence (Table 3; Figure 5). The main difference between the design of the two experiments is that for *Host fitness* we used newly infected amoebas, but for *Symbiont transmission*, we used amoebas that had been infected in a previous vegetative stage to minimize the potential for new symbionts to be acquired from the environment and to maximize our ability to detect horizontal transmission among amoebas. In addition, we infected amoebas for the symbiont transmission experiment at a wider range of MOI (0.3–30) compared to the host fitness experiment (0.6–15) in order to achieve as wide a range of infection prevalence as possible. We found that we were unable to push the maximum of these ranges higher by using a higher MOI for the initial infection.

Combined observations from both experiments suggest that variation in benevolence-based symbiont virulence (Figure 1B) leads to variation in infection prevalence among *P. bonniea* strains infecting amoeba hosts in the longer term. We observed no differences in infection prevalence after the first vegetative stage–social stage cycle (Figure 5A). But relatively malevolent strains bb395 (μ = 36.5, SE = 2.38; bb859–bb395: $p < .001$) and bb433 (μ = 33.8, SE = 2.29; bb859–bb433: $p < .001$) become significantly less prevalent among amoeba hosts compared to the relatively benevolent strain bb859 (μ = 53.5, SE = 2.17) over the course of two vegetative stage–social stage cycles (Figure 5B). A higher rate of horizontal transmission in the relatively benevolent strain compared to relatively malevolent strains appears to contribute to this result (Figure 4).

Candidate virulence factors

The three *P. bonniea* strains used in this experiment do not significantly differ in their per spore bacterial density (Miller et al., 2020). Yet the fact that we observe variation in benevolence-based virulence suggests that bb395 and bb433 possess malevolent virulence factors that bb859 lacks, or bb859 possesses benevolent virulence factors that the other strains do not. Whole genome alignment revealed 41 structural variants greater than 1,000 base pairs between these species (Supplementary Table S3). Of these, the majority (31) were regions unique to bb859, while 9 were present in bb395 and bb433 but absent in bb859. One additional region was found in bb395 and bb859 but was absent in bb433. Our search for potential virulence factors focused on the

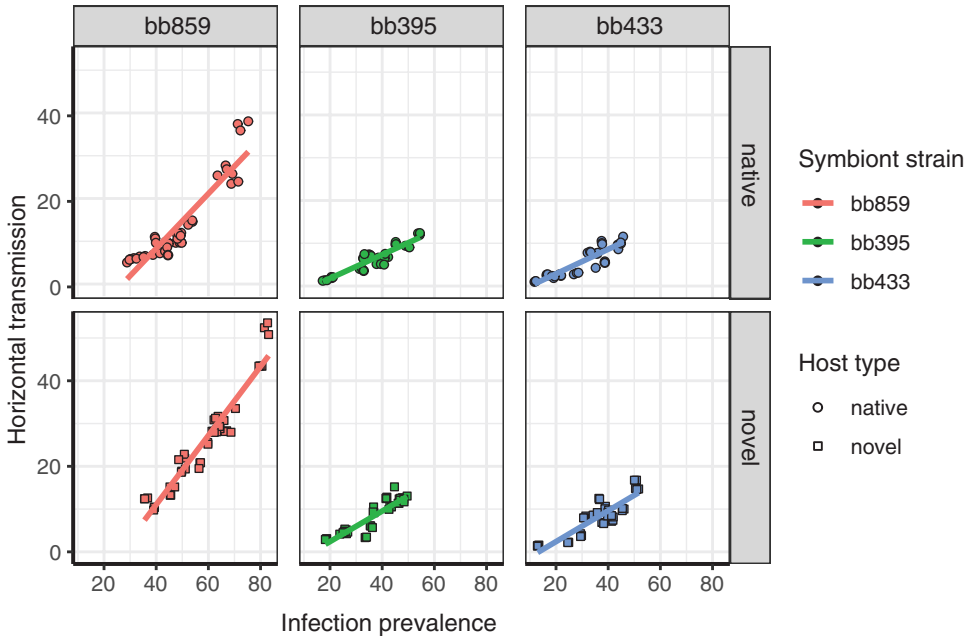

Figure 4. The rate of horizontal transmission was positively correlated with preinfection prevalence and also differed in slope and average infection prevalence among symbiont strains after a second social stage. Infection prevalence was estimated per host–symbiont–MOI sample as the percent of RPF+-infected spores of the preinfected control. Horizontal transmission was estimated by the percent of RFP+-infected spores in the test sample that were also positive for a green membrane dye. These dyed cells were uninfected prior to the experiment, during which they were combined with preinfected (and undyed) cells. There was no significant difference in symbiont transmission among native (top row; lenient definition) and novel (bottom row) hosts.

Table 2. Analysis of deviance table for symbiont transmission with lenient native definition

Variable	χ^2	df	*p*-Value	η^2p	95% CI
Infection prevalence	484.965	1	<.001	0.77	(0.71, 1)
Symbiont strain	149.430	2	<.001	0.43	(0.20, 1)
Host type	2.092	1	.148	0.53	(0.25, 1)
Infection prevalence: Symbiont strain	109.289	2	<.001	0.65	(0.52, 1)
Infection prevalence: Host type	35.991	1	<.001	0.30	(0.17, 1)
Symbiont strain: Host type	9.509	2	.009	0.14	(0.01, 1)

Table 3. Analysis of deviance table for infection prevalence across experiments

Variable	χ^2	df	*p*-Value	η^2p	95% CI
Number of social stages	71.196	1	<.001	0.16	(0.11, 1)
Symbiont strain	17.789	2	<.001	0.02	(0.00, 1)
Number of social stages: Symbiont strain	39.197	2	<.001	0.09	(0.05, 1)

two longest structural variants absent from bb859 that were each approximately 13 kilobases in size and present in strains bb395 (1:108428–122213 and 1:2654181–2667432) and bb433 (1:108428–122213 and 1:2658228–2671479). These variants likely resulted from integration of plasmids as both regions have genes encoding putative plasmid recombinases near the 3′ end.

We also conducted an examination of the structural variants unique to bb859 to identify potential genes that could increase benevolence-based virulence. This search revealed a potential gene in an approximately 6 kilobase structural variant in the genome of strain bb859 (2:710782–715518) that lacks closely related homologs in the other sequenced genomes of *Paraburkholderia* symbionts of *D. discoideum* (Brock et al., 2020).

Discussion

The reduced genome symbiont *P. bonniea* form a facultative symbiosis with *D. discoideum* amoebas. Although *P. bonniea* and two other *Paraburkholderia* symbionts can persistently infect *D. discoideum* in the lab, only a fraction of wild *D. discoideum* hosts appear to be associated with these symbionts (DiSalvo et al., 2015; Haselkorn et al., 2019; Miller et al., 2020). We find evidence that ongoing coevolution is unlikely to affect strain-specific association patterns for *D. discoideum* and *P. bonniea*. When comparing naturally cooccurring (native) and novel host–symbiont strain pairings, both types of associations resulted in similar levels of fitness for hosts and symbionts. Although we observed significant variation in virulence and transmission among *P. bonniea* strains, native hosts did not have enhanced host defenses against *P. bonniea* compared to novel hosts.

Strain-level variation in symbiont virulence: virulence–transmission trade-off

Horizontal transmission of *Paraburkholderia* symbionts was previously assumed, but we demonstrate it for the first time in this study. In addition, despite the limited number of symbiont strains tested, we found evidence that strain-level variation in symbiont virulence affects transmission. Specifically, we found significant variation among *P. bonniea* strains in benevolence-based virulence but not proliferation-based virulence (Figure 3). We observed higher rates of horizontal transmission in the relatively benevolent strain compared to the two relatively malevolent strains (Figure 4). The virulence–transmission trade-off hypothesis posits

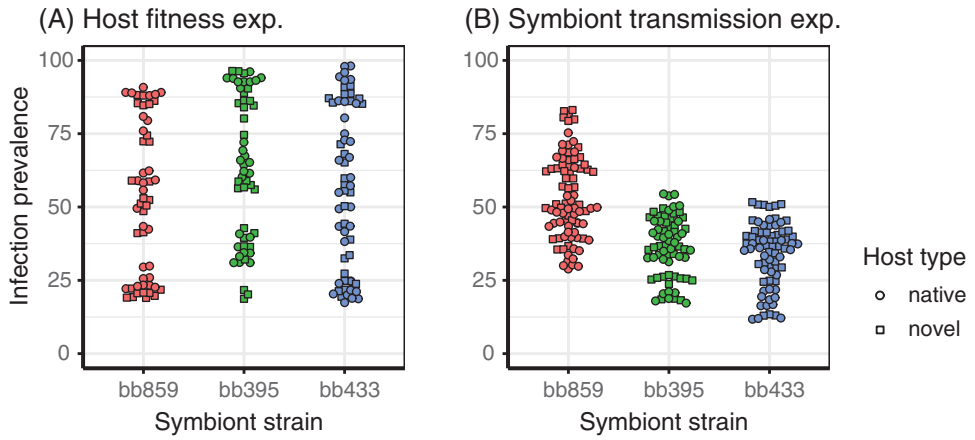

Figure 5. Infection prevalence was significantly different when amoebas were observed one (A) vs. two social stages (B) after infection. Relatively malevolent strains (bb395 and bb433) reach lower infection prevalence among amoeba hosts compared to relatively benevolent strains (bb859) over time.

that if there is a biological link between symbiont virulence and transmission (e.g., if symbionts use host resources to replicate), symbionts should evolve toward a level of virulence where transmission is greatest (Anderson & May, 1982). This hypothesized trade-off has proven difficult to observe for several reasons, including the influences of host range, multiple symbiont infections, and host population structure that can affect host–symbiont interactions in complex ways (Alizon et al., 2009; Leggett et al., 2013). However, recent meta-analyses indicate that there is partial support for the trade-off hypothesis in several biological systems (Acevedo et al., 2019). The three core predictions of the trade-off hypothesis are as follows: (a) within-host–symbiont replication rates have a positive relationship with symbiont virulence; (b) within-host–symbiont replication rates have a positive relationship with symbiont transmission rates; and (c) symbiont virulence and transmission have a trade-off through the negative relationship between symbiont virulence and host recovery rates.

Similar to previous studies (DiSalvo et al., 2015; Garcia et al., 2019; Shu et al., 2018), we estimated host fitness and symbiont transmission at the social group level rather than at the individual amoeba host level. Therefore, our evidence that supports each of these points may be an indirect test of the virulence–transmission trade-off as the original theory is formulated based on investigating symbiont density in individual hosts. However, considering fitness at the population level can facilitate our understanding of how host defense and symbiont virulence evolve (Alizon & Michalakis, 2015). We find support for each of the core predictions of the virulence–transmission trade-off hypothesis. Symbiont infection prevalence had a negative relationship with host fitness (i; Figure 3), and symbiont infection prevalence had a positive relationship with symbiont horizontal transmission (ii; Figure 4). Lastly, variation in symbiont benevolence-based virulence led to increased horizontal transmission of relatively benevolent strains compared to relatively malevolent strains (iii; Figure 4).

Transmission dynamics are an important missing piece of information in further understanding the evolution of the facultative symbiosis between *D. discoideum* and *Paraburkholderia*. Given the persistence of *Paraburkholderia* symbiont infections in the lab and the facultative aspect of the symbiosis itself, we expect these symbionts to transmit among hosts using both vertical and horizontal routes. We did not test for vertical transmission and specifically designed our experiment to use the social stage so that only horizontal transmission was possible. Our results clearly show the presence of significant horizontal symbiont transmission among *D. discoideum* amoebas in the social stage of their life cycle. The coexistence and relative influences of both types of transmission routes are better understood in other amoeba–bacteria symbioses (Herrera et al., 2020).

Lack of variation in host defense: ecology, geography, and experimental approaches

Our results suggest that native hosts of *P. bonniea* do not possess enhanced host defenses against *P. bonniea* compared to novel hosts. These results contrast with previous evidence of host–symbiont coevolution found in *P. hayleyella* and its native hosts (Garcia et al., 2019; Shu et al., 2018). Both *P. hayleyella* and *P. bonniea* have reduced genomes and are sister species to each other (Brock et al., 2020; Noh et al., 2022). The apparent lack of host counteradaptation to *P. bonniea* virulence may be related to the ecology of both host and symbiont. *Paraburkholderia bonniea* is the rarest of *Paraburkholderia* symbionts of *D. discoideum* (DuBose et al., 2022; Haselkorn et al., 2019). Rare encounters between new hosts and *P. bonniea* may not have a significant impact on *D. discoideum* populations, particularly if *D. discoideum* itself is sparsely distributed across soil landscapes. The fitness cost of infection by rare *P. bonniea* may be insufficient for hosts to evolve defenses against it (Anderson & May, 1982).

Another potential reason behind the lack of host defense variation may be because our native and novel hosts were collected from the same geographical locality. In other words, coevolution in this system may occur at the population level rather than at the strain level. We intend to design future experiments to address this question in the *D. discoideum—Paraburkholderia* system. For now, it is unclear whether coevolution within facultative symbioses is more likely between populations than within populations because examinations of strain-level coevolution are limited. Two studies from pea aphids demonstrate the complexity of coevolution in facultative symbioses with tripartite host–symbiont–pathogen or host–symbiont–parasitoid contexts. Facultative *Regiella* symbionts can protect aphid hosts against fungal pathogens, while facultative *Hamiltonella* symbionts can protect aphid hosts against parasitoid wasps. In the first study, genotype-by-genotype interactions affected how protective *Regiella* strains were for host strains against *Pandora* fungi, but native host–symbiont strain pairings were not more protective (Parker et al., 2017). In the

second study, the most virulent *Hamiltonella* strain was also most protective for all host strains against *Aphidius ervi* parasitoids (Niepoth et al., 2018). At the same time, *Hamiltonella* strains were less likely to cause mortality in their native host than in novel hosts. The same study also found that hosts on *Lotus pedunculatus* were more likely to establish symbiosis when newly infected with *Hamiltonella* symbionts compared to hosts from *Lotus corniculatus*, suggesting coadaptation at the population level. *Hamiltonella defensa* is commonly found in hosts on *L. pedunculatus* but is rare in hosts on *L. corniculatus* (Niepoth et al., 2018).

Alternatively, the difference in results between *P. bonniea* and *P. hayleyella* may be due to previous experimental approaches that do not account for host or symbiont fitness as a function of symbiont infection prevalence. We plan to apply our current experimental approach to the relationship between *D. discoideum* and *P. hayleyella*, which is known to cause more detrimental fitness consequences to novel hosts than *P. bonniea* (Miller et al., 2020; Shu et al., 2018). If previous results hold for *P. hayleyella*, it would provide support for the evolution of reduced antagonism in *P. bonniea*. Amoeba hosts experience only a mild reduction in fitness to *P. bonniea* infection compared to *P. agricolaris* or *P. hayleyella* (Miller et al., 2020). Reduced antagonism is a potential outcome of symbiosis that is favored when virulence–transmission tradeoffs are present and new hosts are rare (Johnson et al., 2021; Yamamura, 1993). Both of these conditions appear to hold for *P. bonniea* and support our interpretation that the *D. discoideum*–*P. bonniea* relationship is a stable facultative symbiosis.

Candidate virulence factors for benevolence variation

Although our evidence is limited by the small number of *P. bonniea* strains examined, we identified potential virulence factors that might contribute to the variation in benevolence-based virulence we observed among *P. bonniea* strains. We identified structural variants of interest that are shared between the genomes of bb395 and bb433 but absent in bb859, as well as an additional structural variant that is present in the genome of bb859 but absent in the other two strains.

We identified several candidate genes that may confer increased malevolence to bb395 and bb433 in their shared structural variant. Among these, PB395_00119/PB433_00119 encode a putative member of the peptidase S8 family (also called subtilisin-related peptidases) that are known contributors to the pathogenesis of *Streptococcus pneumoniae* (Ali et al., 2021). Interestingly, there are no closely related homologs in other sequenced *Paraburkholderia* genomes, but proteins with the highest similarity in GenBank are found in *Burkholderia pseudomallei* (MBF3536330.1, 93% identity over the length of the protein) and *Ralstonia solanacearum* (NKA33280.1, 93% identity over the length of the protein), which are, respectively, pathogens of mammals and plants. Potentially, this peptidase may be introduced into *D. discoideum* cells and modify host responses to infection.

Another candidate malevolent virulence factor, PB395_02359/PB433_02360 is a member of the Xenobiotic Response Element family of transcriptional regulators. It is most similar to a homolog in the opportunistic human pathogens of the *Burkholderia cepacia* complex (WP_060080935.1, 80% identity over the length of the protein). In these proteins, the putative DNA-binding domain is at the N terminus of the protein and the C-terminal portion of the protein contains a predicted peptidase domain. This architecture is similar to the *alpR* gene in *Pseudomonas aeruginosa*, which is involved in the pathway of programmed cell death and has been demonstrated as a virulence factor (McFarland et al., 2015).

One gene, in particular, appears to be an interesting candidate for a benevolent virulence factor for strain bb859. PBONN_03430 encodes a putative member of a Type I secretion system known to secrete a wide variety of proteins from Gram-negative bacteria (Spitz et al., 2019). An interesting possibility is that the PBONN_03403 protein may be part of a complex that secretes proteins that modulate activities of *D. discoideum* cells to prevent host cell death and allow bb859 to transmit to other host cells. We are actively working to assess the role of these candidate genes in *P. bonniea* virulence.

Conclusions

Our work demonstrates that ongoing coevolution is unlikely for *D. discoideum* and *P. bonniea* and the system instead represents a stable facultative symbiosis. In this case, naturally cooccurring host and symbiont strain pairings in the system are likely the result of priority effects, and presently unassociated hosts are simply uncolonized. In addition, despite the limited number of symbiont strains tested, we found evidence for a virulence–transmission trade-off without host specificity. Lastly, we identified candidate virulence factors in *P. bonniea* genomes that may be determinants of strain-level variation in benevolence-based symbiont virulence.

Supplementary material

Supplementary material is available online at *Evolution Letters*.

Data and code availability

Full-genome sequences for *P. bonniea* strains are available through NCBI Genomes (txid2152891). Strain bb859 (SAMN09651436) was submitted previously, and bb395 and bb433 were newly submitted (SAMN35686367, SAMN35686368). Data and code supporting this work can be accessed at Dryad at https://doi.org/10.5061/dryad.qz612jmp8 and also at https://github.com/noh-lab/social-infection

Author contributions

S.N. conceived and designed the analysis. S.N. and R.F.P. performed the analysis and wrote the paper. All authors collected the data.

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