

Symbiont-Mediated Protection against Fungal Pathogens in Pea Aphids: a Role for Pathogen Specificity?

Benjamin J. Parker, Chelsea J. Spragg,* Boran Altincicek,* Nicole M. Gerardo

Department of Biology, Emory University, O. Wayne Rollins Research Center, Atlanta, Georgia, USA

Here we show that a bacterial endosymbiont, *Regiella insecticola*, protects pea aphids (*Acyrtosiphon pisum*) from the aphid-specific fungal entomopathogen *Zoophthora occidentalis* but not from the generalist insect fungal pathogen *Beauveria bassiana*. This finding highlights the complex influence of fungi on the dynamics of this economically important agricultural pest.

Symbiotic relationships between invertebrates and vertically transmitted microbes are widespread. One feature of this mutualistic relationship is that symbionts depend on host resources for their own survival and reproduction (1), and theory therefore predicts that in the absence of manipulation of host reproduction, beneficial symbionts must provide a fitness advantage to spread through a host population (2). Studying ecologically relevant traits conferred to hosts by symbionts is critical for understanding host-microbe dynamics, and researchers have therefore searched for fitness advantages of harboring symbionts in a number of systems. Several recent studies have shown that one advantage conferred by some symbionts is protection against pathogens and parasites (3–7). For example, pea aphids (*Acyrtosiphon pisum*), which are a model system for the study of host-symbiont dynamics, are protected against the fungal entomopathogen *Pandora neopaphidis* (Zygomycota: Entomophthorales) by several facultative, vertically transmitted bacteria, including the gammaproteobacteria *Regiella insecticola* (8, 9). Fungi are important natural pathogens of aphids (10) and are used in biocontrol (11, 12), and symbiont-mediated protection to fungi is likely an important factor influencing the population dynamics of aphids and their symbionts. However, aphids encounter several diverse species of fungal pathogens in the wild (13). It is not known if *Regiella*-conferred protection is specific to *Pandora* or if it extends to other species of fungus as well, which would suggest that multiple fungal species are influencing aphid-*Regiella* dynamics. We therefore exposed pea aphids, with and without symbionts, to two additional species of fungal pathogens: *Zoophthora occidentalis* (Zygomycota: Entomophthorales), a highly aphid-specific entomopathogen, and *Beauveria bassiana* (Ascomycota: Hypocreales), a generalist that has been found in a variety of hosts, including species of Coleoptera, Hemiptera, and Diptera (13–15). These fungal species are highly divergent (with some estimates as high as 1,000 million years ago [16]), but both species reproduce by passively releasing spores (conidia) that penetrate the cuticle of a suitable host. Mycelia then colonize the host's body tissue until the death of the host, when new spores are produced and released into the environment.

We used two aphid genotypes, both with and without *Regiella* present (5A, collected in the wild in 1999 near Madison, WI, and subsequently injected with *Regiella* symbionts from an aphid collected in Tompkins County, NY, in 2000; and LSR1, collected on alfalfa near Ithaca, NY, in 1998 with a natural *Regiella* infection and then artificially cleared of symbionts) (17). The use of two aphid genotypes, each with and without *Regiella*, allowed us to

control for effects of aphid genotype on pathogen susceptibility. Before fungal infection, we maintained pea aphids asexually on fava bean (*Vicia faba*) plants in 16 h of light and 8 h of dark at 20°C. We exposed adults to fungus after their final molt (at 9 days of age), as we found that molting shortly after exposure strongly reduces infection probability. After exposure, aphids were kept individually for 4 days on fava bean plants under near 100% humidity, after which the humidity was reduced to 70%. This allowed enough time for fungal penetration of the aphid cuticle, which requires high humidity.

***Zoophthora* (specialist) infection.** We infected aphids with *Zoophthora* by placing them under a “spore shower” (based on references 9, 14, and 18–20). An isolate of *Zoophthora* was obtained from the USDA ARS Collection of Entomopathogenic Fungal Cultures and was grown for 2 weeks on SDAEY plates at 20°C (21). Approximately 15 h before infection, several small pieces of fungal mycelium (3 mm²) were cut with a sterile instrument and placed onto 1.5% tap water agar, which causes the fungus to sporulate. At the time of infection, the agar plates were inverted over a hollow tube for 60 min with aphids at the bottom of the chamber. The agar plates were rotated among treatment groups during infection to ensure that each treatment group was exposed to an equal dose of fungal spores (approximately 16.5 spores/mm²). We included a glass slide in this rotation so that spores could be counted under a light microscope to estimate spore density. Control aphids were handled similarly but were not exposed to fungus.

The *Zoophthora* infection was divided into three blocks conducted several days apart from one another. In each block, we exposed two-thirds of the individuals from each genotype to fungus and kept one-third under identical conditions as a control.

Received 5 November 2012 Accepted 19 January 2013

Published ahead of print 25 January 2013

Address correspondence to Benjamin J. Parker, bparke4@emory.edu.

* Present address: Chelsea J. Spragg, University of Washington, Program in Molecular and Cellular Biology, Seattle, Washington, USA; Boran Altincicek, University of Bonn, INRES-Phytomedicine, Bonn, Germany.

Supplemental material for this article may be found at <http://dx.doi.org/10.1128/AEM.03193-12>.

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doi:10.1128/AEM.03193-12

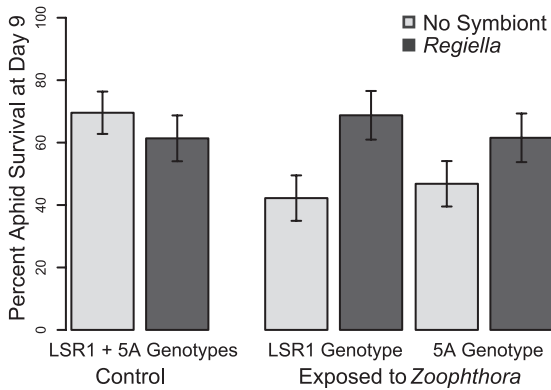


FIG 1 Results of infection with *Zoophthora*. Survival of each aphid was recorded 9 days after infection and is reported as the percent survival of each group. White bars represent aphids with no secondary symbionts ($n = 138$), and gray bars represent aphids that harbored *Regiella* ($n = 131$). Error bars are \pm standard errors.

Half of the aphids of each genotype harbored *Regiella*. Nine days after exposure, we recorded the survival of each aphid. We fit a logistic regression model (a generalized linear model [GLM] with quasibinomial error structure and logit link function) to aphid survival, with symbiont, fungal exposure, and block as fixed effects (22). We used R version 2.11 for our statistical analyses. As expected, aphids exposed to fungus had significantly lower survival than control aphids (odds ratio [OR] = 0.33, 95% confidence interval [CI] = 0.15 to 0.72, $P = 5.8 \times 10^{-3}$). There was also a significant interaction between exposure and symbiont infection on aphid survival (OR = 3.7, CI = 1.2 to 11, $P = 0.021$) but no effect of *Regiella* independently (OR = 0.70, CI = 0.28 to 1.73, $P = 0.44$). This means that in the absence of fungal infection, *Regiella* did not have a significant impact on aphid survival, but it increased survival of aphids exposed to fungus (Fig. 1). The trends were consistent across three blocks, but aphid survival differed across blocks (block 2, OR = 2.6, CI = 1.4 to 4.7, $P = 2.5 \times 10^{-3}$; block 3, OR = 3.4, CI = 1.8 to 6.7, $P = 3.2 \times 10^{-4}$). The trend was

consistent across both aphid genotypes, and there was no significant effect of aphid genotype on survival; it was therefore removed from the model. More aphid genotypes will need to be tested to determine the effect, if any, of aphid genotype on fungal infection outcome. Using this same protocol and these same genotypes, we also confirmed the results of Scarborough et al. (9)—that *Regiella* protects pea aphids from *Pandora*, which, like *Zoophthora*, is an aphid-specific fungal pathogen (see Fig. SA1 in the supplemental material). We also conducted a repeat of the *Zoophthora* infection, using a second pathogen genotype, to ensure that our results were consistent across multiple experiments (see Fig. SA2).

***Beauveria* (generalist) infection.** Cultures of *Beauveria* did not sporulate upon transfer to tap water agar, so we instead made up solutions of *Beauveria* spores (strain GHA, BotaniGard ES) in distilled water. Spores were washed and separated via centrifuge from inert ingredients, and 0.7 μ l of the solution was pipetted onto the dorsal side of the abdomen of each aphid. Half of the aphids of each genotype harbored *Regiella*. We exposed aphids to four different spore doses (0, 25, 250, and 2,500 spores, estimated using a HYPOR KOVA Glasstic hemocytometer). Aphids became infected with *Beauveria* faster than with *Zoophthora*, so for the *Beauveria* infections we recorded the survival status of each aphid at 24-h intervals after exposure, and we analyze these data using a survival analysis (Fig. 2). For the *Beauveria* infection, we took the additional precaution of transferring the symbiont from the 5A line into the LSR1 aphid genotype. Symbiont-free first-instar LSR1 aphids were exposed to the hemolymph of 5A adult aphids that harbored *Regiella* via intrahemocoellic microinjection. Aphids were then kept for at least 10 generations to allow the symbiosis to stabilize and to allow the host lines to adapt to the presence of the bacteria (23). This allowed us to compare survival of genetically identical hosts with two different genotypes of symbionts. Survival data were analyzed using age-specific parametric survival models with a Weibull distribution using the Survival package in R version 2.11. The dose of fungal pathogen exposure had a significant impact on aphid survival (minimal model containing spore dose only, $\chi^2 = 935.96$ on 3 df, $P < 0.0001$). How-

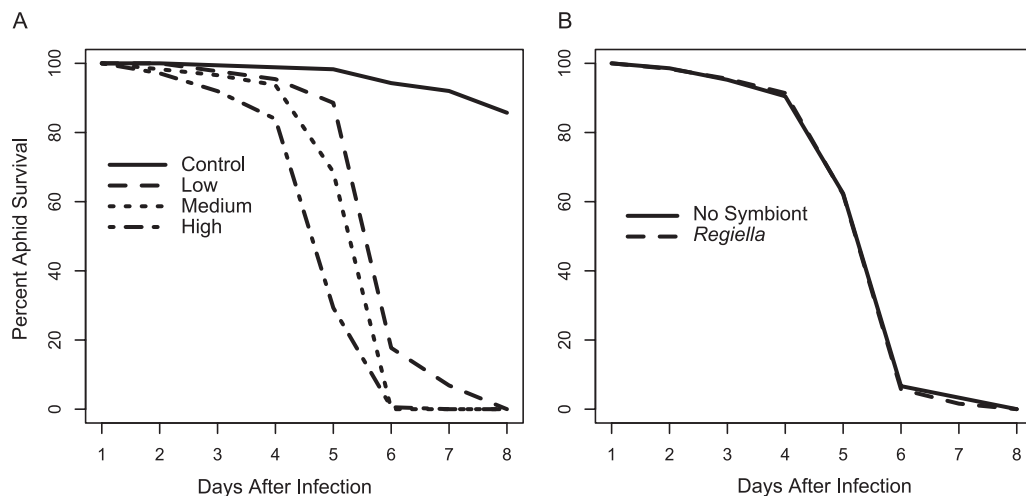


FIG 2 Results of infection with *Beauveria*. Survival of each aphid was recorded every 24 h between 0 and 8 days after infection and is reported as survival curves measured in percent survival of each group. (A) Spore dose. Each line represents a different spore dose (control, 0 spores/aphid; low, 25; medium, 250; high, 2,500), $n = 175$ per dose. (B) Symbiont status. The solid line shows infected aphids without *Regiella* ($n = 210$). The dotted line shows infected aphids that harbored *Regiella* ($n = 315$).

ever, symbiont status had no significant effect on survival, had no interaction with dose, and was removed from the minimal model. This suggests that *Regiella* did not protect aphids from infection with *Beauveria* (Fig. 2). Aphid genotype had no significant effect on survival and was also removed from the minimal model. We repeated this experiment with aphids that harbored two other species of aphid secondary symbionts, *Hamiltonella defensa* and *Serratia symbiotica*, and again found no effect of symbionts on aphid susceptibility to *Beauveria* (see Fig. SB1 in the supplemental material). We also conducted a repeat of the *Beauveria* infection using different spore doses and found no effect of *Regiella* (see Fig. SB2). Lastly, to ensure that this negative result was not due to a sampling effect of *Regiella* genotypes, we conducted an additional experiment where we collected aphids with and without *Regiella* from several geographical locations and assayed their resistance to *Beauveria*. With this experimental design, we are not controlling for host genotype, as each strain used in the experiment will differ both in terms of symbiont and host genotypic background. However, we are able to determine if multiple wild-collected lines with *Regiella* are, on average, more resistant to *Beauveria* than wild-collected lines without symbionts. We found significant variation in resistance to *Beauveria* among aphid genotypes, but no effect of harboring *Regiella* symbionts (see Fig. SC in the supplemental material), suggesting that our results are not due to a lack of diversity in symbiont genotypes.

Together, these data suggest that *Regiella* symbionts confer protection against several specialist fungal pathogens but not against a generalist pathogen. *Regiella* frequencies vary among aphid populations (24, 25), and researchers have tried to determine the factors that influence symbiont frequencies as a way of better understanding this model host-microbe interaction. Variation in *Regiella* frequency is explained in part as a balance between the benefits of protection from fungal pathogens and the costs of harboring bacteria. Our results suggest that several species of fungal pathogens may be driving this interaction but that *Regiella* is not beneficial against all species of fungi. In addition, these fungal pathogens are used in aphid biocontrol (11, 26, 27), and our results suggest that symbiont-mediated protection against pathogens may be an important consideration when selecting and developing biocontrol agents.

In general, researchers are working to develop an understanding of how evolution acts on the alternative defenses that organisms have to protect themselves from pathogens and parasites (28). One possible explanation for the pattern of symbiont-mediated protection observed here is that *Regiella* protection has evolved in response to pressure from individual species of fungal pathogens. A second possibility is that protection in this system has evolved in response to fungal pathogens that specialize on aphids but not to generalist insect pathogens, perhaps due to broad differences found in the infection strategies of generalists versus specialists (29, 30). Pathogen specificity has been shown to be an important factor influencing host-enemy interactions (31), and we therefore highlight the relative specificity of these pathogens as a potential explanation for the pattern of protection observed in this system, but clearly more data are needed. It is possible that close coevolution between aphid specialists and *Regiella* is needed to develop or maintain protection conferred by symbionts.

ACKNOWLEDGMENTS

Thierry Lefèvre, Alice Laughton, Eleanore Sternberg, Anand Bhardwaj, and four anonymous reviewers provided valuable assistance with the manuscript. We especially thank Richard Humber, Karen Hansen, and the USDA ARS Collection of Entomopathogenic Fungal Cultures, who provided cultures of *Zoophthora*.

This research was supported by NSF IOS-1025853 to N.M.G. B.A. was supported by a DFG grant (AL902/4-1), and B.J.P. is supported by an NSF Graduate Research Fellowship.

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