Exposure to cuticular bacteria can alter host behavior in a funnel-weaving spider

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Abstract

Contact with environmental microbes are arguably the most common species interaction in which any animal participates. Studies have noted diverse relationships between hosts and resident microbes, which can have strong consequences for host development, physiology, and behavior. Many of these studies focus specifically on pathogens or beneficial microbes, while the benign microbes, of which most of the majority of bacteria could be described, are often ignored. Here, we explore the nature of the relationships between the grass spider Agelenopsis pennsylvanica and bacteria collected from their cuticles in situ. First, using culture-based methods, we identified a portion of the cuticular bacterial communities that are naturally associated with these spiders. Then, we topically exposed spiders to a subset of these bacterial monocultures to estimate how bacterial exposure may alter 3 host behavioral traits: boldness, aggressiveness, and activity level. We conducted these behavioral assays 3 times before and 3 times after topical application, and compared the changes observed in each trait with spiders that were exposed to a sterile control treatment. We identified 9 species of bacteria from the cuticles of 36 spiders and exposed groups of 20 spiders to 1 of 4 species of cuticular bacteria. We found that exposure to Dermacoccus nishinomiyaensis and Staphylococcus saprophyticus was associated with a 10-fold decrease in the foraging aggressiveness of spiders toward prey in their web. Since bacterial exposure did not have survival consequences for hosts, these data suggest that interactions with cuticular bacteria, even non-pathogenic bacteria, could alter host behavior.

Key words: Agelenopsis pennsylvanica, aggressiveness, Araneae, cuticular bacteria, personality
Animals are constantly subject to microbes from their environment, many of which develop important relationships with their host, altering host physiology, health, and behavior. Extensive research has probed the myriad roles that certain microbes play in the functioning of the endocrine, digestive, immune, circulatory, and nervous system of their hosts (McFall-Ngai et al. 2013). Although interactions between hosts and environmental bacteria have classically been overlooked in the field of behavioral ecology, contemporary research has linked members of host-associated microbial communities with important host behaviors like reproduction, habitat selection, and foraging (Grenham et al. 2011; Ezenwa et al. 2012). Most of these studies focus on either pathogenic or beneficial microbes that reside in the host gut. For example, Lactobacillus gut bacteria attenuate anxiety-related behaviors in both mice (Bravo et al. 2011) and zebrafish (Davis et al. 2016), whereas infection by Trichuris muris increases the expression of these behaviors (Bercik et al. 2010). In German cockroaches, exposure to commensal gut microbiota stimulates the production of volatile carboxylic acids which increases aggregation tendencies, indicating that gut bacteria may act as a mediator of insect–insect interaction (Wada-Katsumata et al. 2015). Fewer studies, however, have sought to link hosts’ behavioral traits with exposure to potentially benign microbes, which constitute the majority of bacteria with which an animal interacts (Nishiguchi et al. 2008; Dinan et al. 2015).

Host cuticles represent the first physical barrier between the body and exogenous microbes. Although many members of the skin microbiome are considered benign and unlikely to influence important host traits (Grice and Segre 2011), evidence suggests that some cuticular bacteria can play important roles in host behavior. For instance, the harvester ant Pogonomyrmex barbatus uses colony-specific cuticular microbial communities to recognize nest mates (Dosmann et al. 2016). Research on the social spider Stegodyphus dumicola has revealed nuanced relationships between hosts and cuticular bacteria (Keiser et al. 2016a), where increases in cuticular bacterial load (i.e., abundance) can alter colonies’ collective foraging behavior (Keiser et al. 2016b). Taken together, these examples illustrate that seemingly benign cuticular bacteria can play a role in host’s behavior.

Here, we investigate whether, and to what degree, exposure to cuticular bacterial can alter important behaviors and survivorship in the grass spider Agelenopsis pennsylvanica. Although A. pennsylvanica are predominantly solitary, spiders may share cuticular bacteria via shared environments (e.g., silk or soil) or during interactions with conspecifics (e.g., antagonistic interactions or mating). We collected spiders from urban and suburban habitats and collected bacteria from their cuticles to (1) characterize some of bacteria with which these animals likely interact, and (2) use these cuticles to (2) expose spiders to these bacteria and determine if spiders alter their behavior.

**Materials and Methods**

**Collection and maintenance**

*Agelenopsis pennsylvanica* (*N* = 120) both male and female spiders were collected by locating their funnel-shaped webs in bushes and hedges in Pittsburgh, PA in Oakland, Squirrel Hill, and Lewisburg suburbs. One 2-week-old cricket was placed at the opening of the funnel using tweezers. When the spider emerged from the back of the web to attack the cricket, the spider was quickly scooped into a plastic container and transferred directly to a sterile 50 mL conical tube. Spiders were transported back to the laboratory and isolated into round (diameter = 12 cm), opaque, sterile plastic housing containers. Spiders were maintained in an environment chamber at 25°C and a 16:8 h light:dark cycle. Spiders were restricted to a maintenance diet of one 2-week-old cricket per week and were provided water by misting the web with a spray bottle. We estimated individuals’ body size by measuring their prosoma width (millimeter) and body mass (gram) with digital calipers and a digital scale.

**Bacterial isolation and identification**

One week after collection and storage in a sterile housing container, 36 spiders were chosen haphazardly and each transferred to a sterile 15 mL conical tube containing 1 mL sterile LB broth (Fisher Scientific, Asheville, NC, USA), then subsequently shaken by a vortex (700 × g) for 10 s. Spiders were then returned to their original housing container using forceps. Four 10-fold serial dilutions were made from this stock solution and 100 μL of each dilution was spread onto LB agar plates. Plates were incubated at room temperature for 24–72 h, until bacterial growth was evident. Unique individual bacterial colonies (by color and morphology) were re-streaked using sterile inoculating loops onto new LB agar plates 3–4 times to ensure that cultures were monospecific. Although LB agar is a nutrient rich medium, not all environmental bacteria are able to readily grow under its conditions. Thus, only a subset of the spiders’ cuticular bacterial community were likely to be cultured in this way.

Bacterial identification was performed by PCR amplification of a 1500 bp region of the prokaryotic 16S ribosomal RNA gene using illustra PuReTaq Ready-To-Go PCR Beads (GE Healthcare Life Sciences, Pittsburgh, PA, USA) and sent for sanger sequencing (GeneWiz, South Plainfield, NJ, USA). See the Appendix for primer details. Bacterial identification was determined by manually aligning sequences in FinchTV BLAST software (Geospiza, Inc., Seattle, WA, USA). We used a cutoff of ≥99% identity for identifications at the species level, and ≥97% identity for identifications at the genus level. All bacterial strains were stored at −80°C in 25% glycerol and are available upon request.

**Behavioral assays**

We performed 3 behavioral assays to characterize each spider’s behavioral phenotype before versus after experimental exposure to bacteria: boldness, activity, and aggressiveness. These represent important behaviors for the natural history of *Agelenopsis* spiders that are regularly subject to ongoing natural and sexual selection (Berning et al. 2012; e.g., antipredator behavior and mating; Riechert and Hedrick 1990). Each assay was conducted 3 times per spider before and after bacterial exposure for a total of 6 trials (2 sets of 3 trials). Behavioral assays were all performed on the same day in the following order: boldness, activity, and aggressiveness. Spiders that were vortexed to collect bacterial samples were not included in the subsequent behavioral assays or bacterial exposures.

**Boldness assay:** Boldness is a behavioral trait defined as an individual’s propensity to engage in risky behavior (Sloan Wilson et al. 1994) and is often measured as an individual’s latency to resume normal activity following an antagonistic stimulus (Riechert and Hedrick 1993). To assess spiders’ boldness, we opened the lid to spiders’ home containers and allowed a 30-s acclimation period, after which 2 rapid puffs of air were administered to the anterior prosoma using a plastic transfer pipette bulb. This causes the animal to either flee rapidly from the stimulus or to perform a “death feign” and remain motionless. We recorded whether or not the spider fled from the stimulus, and the latency in seconds for the spider to resume...
normal movement about the web. Bolder spiders resume activity faster, and shyer spiders remain motionless for longer. If the spider had not resumed activity within 300 s, the trial was terminated and the spider was scored with the maximum value of 300 s.

Activity assay: To assess spiders’ activity level, we transferred the spider to a clean, plastic container (diameter = 12 cm) and placed a black plastic dish over the spider. After 30 s acclimation, the black dish was removed and the proportion of time that each spider spent moving around the container was measured in seconds over a 300 s period using a stopwatch.

Aggressiveness assay: To assess spiders’ foraging aggressiveness toward prey items, we removed the spider’s home container lid and allowed a 30 s acclimation period. After which, a single 2-week-old cricket was placed in the center of the web using tweezers. We measured the latency for the spider to attack the cricket. More aggressive spiders attack prey faster. If the spider did not attack the cricket within 300 s, the assay was terminated and the spider was scored with the maximum value of 300 s.

Bacterial liquid monocultures were grown by picking an individual bacterial colony from an LB plate and placing it in 3 mL of sterile LB broth. After 24 h shaking at room temperature, we centrifuged the solution at 4,000 × g for 5 min, removed the supernatant, and suspended the bacteria in 1 mL of 0.005% Triton-X solution (Sigma Aldrich, St. Louis, MO, USA). This surfactant allows liquids to be applied topically to spiders’ hydrophobic cuticles. In 2 side experiments, we inoculated LB plates with 40 µL of bacterial monocultures in either phosphate-buffered saline or 0.005% Triton-X and found that Triton-X does not reduce bacterial growth. Then, we exposed 5 adult A. pennsylvaniae to 0.005% Triton-X and saw no mortality over the remainder of the experiment (data not shown). We measured the optical density of each monoculture at 600 nm and diluted each solution to an optical density of 1 OD in 0.005% Triton-X. Spiders were randomly separated into either the bacterial exposure (N = 41) or control group (N = 43). To expose spiders to their assigned cuticular bacteria, 5 µL of a bacterial monoculture solution (i.e., approximately 4 × 10^6 CFUs) was applied to the spider directly above the pedicel using a micropipette. Similar methods have been used previously to increase cuticular bacterial loads on spiders (e.g., Keiser et al. 2016a, 2016b). The spiders in the bacterial exposure group were only inoculated with 1 of the 4 isolated strains, resulting in experimental groups with approximately 10 spiders per strain. For spiders in the control groups, 5 microliters of autoclaved 0.005% Triton-X solution was administered atop the pedicel. Spiders were then placed back into their housing container until behavioral assays recommenced 7 days later. The survival of all spiders was monitored for the following 3 weeks.

Statistical analyses
We calculated the change in average behavioral trait values (boldness, aggressiveness, and activity) by subtracting the average value of the 3 post-exposure trials from the average value of the 3 pre-exposure trials. Thus, because boldness and aggressiveness are latency values, a positive calculated value conveys an increase in aggressiveness or boldness after bacterial exposure while a negative value denotes a decrease. A positive value in change in activity, however, represents a decrease in activity post exposure. We compared the change in each behavioral value between exposed spiders and corresponding control spiders using generalized linear mixed models (identity link function; after verifying model assumptions) with the following independent variables: spider sex, bacterial exposure (bacteria vs. control), and spider body condition. Body condition is estimated as the residual values from a regression of individuals’ body mass and prosoma width (Jakob et al. 1996). Spider survival (alive/dead) after 3 weeks was analyzed with nominal logistic regressions with the same independent variables as above. The collection location in the city from which spiders were collected was included as a

### Table 1. Bacteria collected from the cuticles of A. pennsylvaniae spiders

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>Characteristics</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td><em>Dermacoccus nishinomiyaensis</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Catalase positive, Gram-positive, aerobic. Found widely in environment including: water, soil, and occasionally on mammalian skin.</td>
<td>Stackebrandt et al. (1995)</td>
</tr>
<tr>
<td><em>Serratia marcescens</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Catalase positive, Gram-negative, facultative anaerobe, motile. Found in many infections including respiratory tract infections, urinary tract infections, and wound infections. Some strains are facultatively pathogenic in insects.</td>
<td>Hejazi and Falkiner (1997)</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Catalase positive, Gram-negative, facultative anaerobe, non-motile. Common in soil and plants; has also been shown to elicit an immune response in arthropods.</td>
<td>(Insua et al. 2013; Yang and Chen 2016)</td>
</tr>
<tr>
<td><em>Staphylococcus saprophyticus</em>&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Catalase positive, Gram-positive, aerobic, and motile. Found in normal flora of female genital tract, can cause urinary tract infections. Common pathogen that is often found in the environment.</td>
<td>Raz et al. (2005)</td>
</tr>
<tr>
<td><em>Staphylococcus gallinarum</em></td>
<td>Catalase positive, Gram-positive, aerobic, and motile.</td>
<td>Yu et al. (2008)</td>
</tr>
<tr>
<td><em>Pseudomonas psychrotolerans</em></td>
<td>Catalase positive, Gram-negative, aerobic, motile. Most often found in soil and water.</td>
<td>Hauser et al. (2004)</td>
</tr>
<tr>
<td><em>Cartobacterium sp.</em></td>
<td>Catalase positive, Gram-positive, aerobic. Common soil bacteria and can be a plant pathogen.</td>
<td>Funke et al. (2005)</td>
</tr>
<tr>
<td><em>Pseudomonas sp.</em></td>
<td>Catalase positive, Gram-negative, aerobic, and motile. Depending on sp., can be pathogenic in plants, arthropods or humans. Mainly found in water and plants.</td>
<td>Holt et al. (1994)</td>
</tr>
<tr>
<td><em>Dermacoccus sp.</em></td>
<td>Catalase positive, Gram-positive, aerobic, and non-motile. Rarely pathogenic, often found in water and human flora.</td>
<td>Holt et al. (1994)</td>
</tr>
</tbody>
</table>

<sup>a</sup>Denotes bacteria that were used to experimentally expose spiders.
random effect in all models. All statistical analyses were performed in JMP Pro version 12.1.

Results

Bacterial identification
We identified 9 unique bacteria from the cuticles of A. pennsylvanica (Table 1). Many of these were common environmental bacteria found in soil, water, or on plant surfaces. Three of our identified bacteria had previously been described as pathogenic in arthropods (Serratia marcescens, Pseudomonas, and Klebsiella pneumonia; Table 1).

Changes in spider behavior
The sex of the host spider was not associated with changes in any behavioral traits (all $P > 0.11$; Table 2). Spiders’ body conditions were associated with their change in behavior in some exposure cases (e.g., when spiders were exposed to Staphylococcus saprophyticus and Klebsiella pneumonia; Table 2). Exposure to Dermacoccus nishinomiyaensis ($\chi^2 = 5.62, P = 0.01$; Figure 1A) and S. saprophyticus ($\chi^2 = 9.06, P = 0.003$; Figure 2) was both associated with over a 10-fold decrease in spiders’ foraging aggressiveness toward prey whereas control spiders showed no change in aggressiveness. Spiders, on average, showed an increase in their latency to resume activity (i.e., became less “bold”) over time in the absence of bacterial exposure ($F_{1,12,6} = 8.87, P = 0.01$). However, spiders that were exposed to K. pneumoniae showed no difference in boldness after bacterial exposure (Figure 3) while spiders that were exposed to D. nishinomiyaensis showed a increase in latency to resume activity ($\chi^2 = 6.44, P = 0.01$; Figure 1B). Spiders that were exposed to bacteria did not differ in their survival rates 3 weeks post exposure when compared with control spiders (all $P > 0.22$), though males were more likely to have died compared with females, regardless of treatment group ($\chi^2 = 11.29, df = 1, P = 0.0008$).

Discussion

Countless studies have identified important relationships between pathogenic and beneficial microbiota and the physiology and health of their host. Fewer studies, however, have identified links between environmental, exogenous microbes and hosts’ behavioral traits. This is despite the fact that these bacteria interact with hosts constantly. We investigated here whether the behavior of the grass spider A. pennsylvania was altered by being exposed to an increased abundance of bacteria collected from conspecifics’ cuticles in situ. We focused on 3 important host behavioral traits (boldness, aggressiveness, and activity level) and found that exposure to cuticular bacteria had the strongest influences on spiders foraging aggressiveness toward prey, though we found no evidence that increased cuticular bacterial load was actually harmful to spiders, at least in terms of survival rates.

The 9 bacteria collected from spiders’ cuticles were mostly environmental bacteria often found in soil, water, or on plant surfaces. Thus, spiders likely acquire these microbes while moving around their environment during dispersal, web construction, foraging, and possibly mating. Notably, the widespread arthropod pathogen S. marcescens was isolated from 2 different spiders. We propose that future studies might examine if external exposure to pathogenic bacteria could lead to invasion into the host body cavity and cause infection. It is possible that cuticular bacteria might invade a spider’s body through the spiracles (Bassett et al. 2000) or via grooming (Forster 1977). Pseudomonas was also collected from 2 spider cuticles, and the pathogenicity of P. aeruginosa has been confirmed in a wolf spider (Gilbert et al. 2016). A study by Gilbert and Uetz (2016) further demonstrated that P. aeruginosa can be horizontally transmitted during spider mating. Thus, there is impetus to believe that contact between conspecifics during mating, parental care, or territorial interactions could result in the transmission of both

Notes: Significant effects are denoted with an asterisk. Empty values represent effects where comparisons could not be made due to uneven distribution of male and female spiders in that group.
We found that cuticular exposure to *D. nishinomiyaensis* and *S. saprophyticus*, 2 common soil bacteria, were associated with over a 10-fold decrease in spiders’ foraging aggressiveness toward prey. Spiders that were exposed to these 2 bacteria attacked prey, on average, over 60 s more slowly than control spiders. Neither bacterium is known to produce secondary metabolites which could plausibly cause such effects. Regardless of the mechanism, a reduction in foraging aggressiveness is likely to have fitness consequences in these animals *in situ*. Members of the genus *Agelenopsis* create sheet-like non-sticky capture webs and therefore rely on high burst speeds to capitalize on time-sensitive foraging opportunities (Turnbull 1965; Hedrick and Riechert 1989). If such reduced burst speeds translate to poor locomotor performance, this could limit spiders’ ability to forage and defend their territory from intruding conspecifics (Pruitt and Husak 2010). Further, exposure to *D. nishinomiyaensis* and *K. pneumoniae* resulted in either an increase in spiders’ latency to resume activity after an aversive stimulus or no change relative to control spiders, respectively. If these cuticular bacteria can enter the body via grooming, resulting in activation of their immune system (Zhukovskaya et al. 2013), which could explain the spiders latency to resume activity, future studies should test whether spiders increase grooming behavior after exposure and if there is an increase in bacterial CFUs in the gut/hemolymph after grooming. Although we found no evidence that bacterial exposure altered spider survivorship in the laboratory, we reason that stable abiotic conditions and ample access to prey may have reduced the opportunity to observe effects on spider performance. Although there is obvious still much to discover in these system, data like these hint that interactions between spiders and their microbes are likely to have large effects on host traits.

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Appendix

Table A1. PCR primer information

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer</th>
<th>Forward sequence (5' → 3')</th>
<th>Reverse sequence (5' → 3')</th>
<th>Amplipon size (bp)</th>
</tr>
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<tbody>
<tr>
<td>16S ribosomal subunit</td>
<td>Illustra PuReTaq Ready-To-Go PCR Bead</td>
<td>GAGTTTGATCCTGGCTCA</td>
<td>ACGGCTAACTTGGTTACGACT</td>
<td>143</td>
</tr>
</tbody>
</table>