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Microbial Associates of the Southern Mole Cricket (*Scapteriscus borellii*) are Highly Pathogenic

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Running Head: Mole cricket microbes

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Abstract

We report the isolation and identification of seven bacterial strains and one fungal strain from dead and diseased *Scapteriscus borellii* mole crickets collected from a golf course in southern California. Using 16S and 18S rRNA gene sequence analysis we identified the microbes as *Serratia marcescens* (red), *S. marcescens* (white), *S. marcescens* (purple), *Achromobacter xylosoxidans*, *Chryseobacterium sp.*, *Ochrobactrum anthropi*, *Tsukamurella tryosinosolvens*, and *Beauveria bassiana.* We performed a dose response curve for each of these cricket-associated microbial strains (except *T. tryosinosolvens)* and two other strains of *S. marcescens* (DB1140 and ATCC 13880). We found that all of these microbes except *O. anthropi* were highly pathogenic to *D. melanogaster* compared to the other strains of *S. marcescens*. Injecting the mole cricket associated strains of *Serratia* into flies killed all infected flies in $\leq$ 24 hours. For all other strains, the median time to death of injected flies varied in a dose-dependent manner. *In vivo* growth assessments of these microbes suggested that the host immune system was quickly overcome. We used disease tolerance curves to better understand the host-microbe interactions. Further studies are necessary to understand in mechanistic detail the virulence mechanisms of these mole cricket associated microbes and how this association may have influenced the evolution of mole cricket immunity.

Key words: Mole crickets, *Scapteriscus borellii*, *Serratia*, *Beauveria*, environmental microbiology

1. Introduction

Mole crickets in the family of Gryllotalpidae are considered major pests of turfgrass and other economically important row crops worldwide (Nickle and Castner 1984). More than 90 species of mole crickets have been described but only a few of them have been reported as crop pests (Dillman et al. 2014; Frank 2009; Frank and Parkman 1999; García 2006; Nickle and Frank 1988; Walker 1984; Walker and Nickle 1981). Mole crickets are distributed throughout temperate, subtropical and tropical regions globally (Frank 2009; Frank and Parkman 1999). The short-winged mole cricket, *Scapteriscus abbreviatus*; the southern mole cricket, *S. borellii* (previously known as *S. acletus*), and the tawny mole cricket, *S. vicinus*, are all native to South America, and were unintentionally introduced into the southeastern US in the early 20th century (Walker and Nickle 1981). These mole crickets have since spread and have recently been reported in Arizona, California, and Mexico (Dillman et al. 2014; García 2006; Nickle and Frank 1988; Walker 1984).

Many soil-dwelling insect pests such as mole crickets pose substantial problems for pest management (Jackson et al. 2000). Mole crickets are hidden under the soil and are difficult to detect until they cause substantial visual damage to the crops or turf (Walker and Nickle 1981). Soil systems are complex and delicate such that chemical control of mole crickets and other pests is often ineffective without the use of high amounts of pesticide and repeated applications (Xia et al. 2000). The successful control of mole crickets using biological control agents, particularly entomopathogenic nematodes (EPNs) such as *Steinernema*

62 *carpocapsae* and *St. scapterisci*, in the early 80s and 90s in Florida has
63 increased research interests in multifaceted interactions between hosts and
64 pathogens (Akhurst and Dunphy 1993). Despite successes in controlling mole
65 cricket populations using natural means, there remains strong evidence of
66 invasive spread, especially in the western US (Arakelian 2008; Dillman et al.
67 2014; Frank 2009; Walker and Nickle 1981). Difficulties in sampling (mostly due
68 to subterranean habitat) and laboratory rearing make mole crickets a difficult host
69 to study, and little is known about their immune system or their associated
70 microbes.

71 While *St. scapterisci* has been successfully used to control mole cricket
72 populations in the field, our understanding of the host-pathogen interaction during
73 the pathogenesis of *S. borellii* remains limited. Understanding these interactions
74 in mechanistic detail may help prevent the continued spread of mole cricket pests
75 and promote the successful utilization of *St. scapterisci* and other biocontrol
76 agents. To increase our understanding of host-pathogen interactions between
77 mole crickets, nematode parasites, and bacterial pathogens, we collected *S.*
78 *borellii* adults from a golf course in southern California during the cricket mating
79 seasons (April-August) of 2015 and 2016. In maintaining them, we observed
80 disease and rapid death in ~15% of the *S. borellii* we had collected. Here, we
81 report the isolation and identification of microbes associated with diseased and
82 dead *S. borellii* that were collected from the field. We assessed the virulence of
83 these microbial isolates in adult *Drosophila melanogaster*. Here we report the

isolation and characterization of cultivable aerobic bacteria and fungi associated with *S. borellii*, and assess their pathogenicity and *in vivo* growth.

2. Materials and Methods

2.1. *Microbes associated with Scapteriscus borellii mole crickets*

Scapteriscus borellii adults were collected twice a week from the Rio Hondo golf course in Downey, California between April to August of 2015 and 2016. Modified versions of a portable acoustic sound traps were used to collect crickets with varying complexity from a sophisticated inverted umbrella design to a simple flat bed sheet stapled to a plastic box as previously described (Dillman et al. 2014). Female crickets were attracted using electronic emitters (Ulagaraj and Walker 1973) that mimic the sound of the song of male mole crickets produced in the evenings during the spring and summer months to attract females for mating (Ulagaraj 1976). Each emitter contained a sound-synthesizer, a sound controller, an amplifier, a speaker, and a 12v DC rechargeable power source as previously described (Dillman et al. 2014). The sound-synthesizer was a computer chip programmed with the song of *S. borellii*. Male crickets were tracked while they were singing and dug out from the tunnels with the help of mini shovels. This process was continued 1 to 1.5h each evening and mole crickets were collected in a plastic shoe box (34.6 cm x 21 cm x 12.4 cm) containing 5-6 adults to avoid potential fighting, as they do not like to be close to each other. Trapped adult crickets were collected and securely transported to a quarantined insectary at the University of California Riverside (CDFA permit

number 3144). Each individual adult was marked and kept in a container fabricated from PVC pipe and polystyrene Petri dish lids (Fisher #0875712). A piece of schedule 40 PVC coupling pipe 8.3 cm long with an outside diameter of 8.9 cm was capped on each end with a Petri dish lid, 9.1 cm x 0.9 cm (outside diam x height). The top lid was ventilated by drilling 6-8 holes while an undrilled lid was used as the bottom. Each container was filled with ~250 cc of clean, autoclaved and lightly moistened sand. The Petri dish lids, serving as the top and bottom, were secured to the PVC pipe with #64 rubber bands. Each individual was fed twice a week and any old food present at the next feeding was removed using sterile tweezer. Any container with dry sand was lightly moistened with tap water as needed throughout the rearing process. All the *S. borellii* crickets were maintained at 25°C and 60% humidity. Freshly dead and diseased *S. borellii* were collected for this study.

Every day, freshly dead and diseased *S. borellii* were collected and surface sterilized with 75% ethanol before dissection. Diseased adults were identified as still alive but possessing the following characteristics: non-feeding, reduced mobility, and decreased response to touch stimuli. The crickets were symmetrically bisected with the help of sterilized dissection scissors. Microbial samples were taken from the interior of the thorax region using a sterilized inoculation loop and were streaked on each of three types of plates: lysogeny broth (LB) nutrient agar plate, LB plate supplemented with carbenicillin (50 mg/L), and LB plate supplemented with carbenicillin (50 mg/L) and kanamycin (50 mg/L). Mole crickets are a known natural host for *St. scapterisci* and other EPNs

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(Nguyen and Smart 1990; Nguyen and Smart 1992). Antibiotics were used primarily to try to isolate EPN associated bacterial isolates i.e. *X. innexi* or X. *nematophila* if present in mole crickets. We did not find any species of *Xenorhabdus* or *Photorhabdus* associated with these mole crickets although *S. marcescens* has been previously identified as a symbiont of rhabditid EPNs (Torres-Barragan et al. 2011). The plates were incubated at 28 °C overnight and distinct morphological colonies were identified based on their color and size. Each distinct colony from each plate was re-isolated and streaked on corresponding plate for obtaining the pure culture. Finally, an overnight culture was prepared from all the purified colonies and stored at -80°C in 20% glycerol stock. Periodic tap water samples were cultured using the above mentioned conditions and were not found to have bacterial growth as potential contamination. All the bacterial work was carried out under an aseptic conditions. This process was repeated on 30 freshly dead and 10 diseased *S. borellii* crickets.

Single colonies of bacteria strains were selected and grown in 5 mL liquid LB overnight agitated at 200 RPM at 30°C in a shaking incubator. 2 mL of liquid culture were spun down at 15,000 RPM for 30 seconds using a tabletop centrifuge. Supernatant was discarded and the pellet was vigorously resuspended with 500 µL of 2% Cetyltrimethylammonium bromide preheated to 65°C. Samples were incubated at 65°C for 15-30 minutes. Next 500 µL of 24:1 Chloroform:Isoamyl Alcohol was added and thoroughly mixed by inversion (>2 min). Samples were then spun at 7,000 g for 10 minutes in a tabletop centrifuge.

Upper aqueous supernatant was pipetted into fresh 1.5 mL tubes then 3M Sodium Acetamide (1/10 volume) was added followed by Isopropanol (1 volume). After a few gentle inversions the samples were centrifuged at 3,000 g for 2 minutes, then the supernatant was decanted. To wash the DNA 500 µL freshly prepared cold 70% ethanol was added followed by an additional spin (3,000 g, 2 min), then decanted. The pellet was dried at 65°C for 2 minutes then suspended in 250 µL DEPC water. 1 µL was carried forward as template for PCR using the following primers to amplify 16S/18S sequences (36, 37): 16S 8F – 5′-AGAGTTTGATCMTGGCTCAG-3′, 16S 1492R – 5′-GGTTACCTTGTTACGACTT-3′, 18S - 5′-AAACGGCTACCACATCCAAG-3′, 18S - 5′-GTACAAAGGGCAGGGACGTA-3′.

Amplicons were sequenced with Sanger BigDye chemistry on an Applied Biosystems 3730xl DNA sequencer, trimmed using Chromatogram Explorer v. 3.2 and species identification made by best hits to the 16S database using BLASTN. All sequences were combined into a single file in FastA format and uploaded to www.phylogeny.fr to perform multiple alignments, trimming, and phylogenetic inference (Anisimova and Gascuel 2006; Dereeper et al. 2008; Dereeper et al. 2010;). Sequences were aligned using MUSCLE (Edgar 2004; v3.8.31) with default settings and trimmed using Gblocks (Castresana 2000; v0.91b) with the following parameters: minimum length of a block after gap removal: 10, no gap positions were allowed in the terminal positions in the alignment, all segments with contiguous nonconserved positions bigger than 8 were rejected, minimum number of sequences for a flank position: 85%. A gene

tree from the 16S sequences was created by PhyML program (Guindon and Gascuel 2003; v3.1/3.0 aLRT) using the following settings: Model: HKY85, Statistical test: aLRT, Number of categories: 4, Gamma: 0.725, Invariable sites: 0.369, Remove gaps: enabled, 100 bootstraps. The tree was visualized using Figtree (Rambaut and Drummond 2009; v. 1.3.1). The 16S and 18S sequences were submitted to Genbank and assigned accession numbers MF103678-MF103684.

2.2. *Virulence of microbes associated with* S. borellii *mole crickets*

All the pathogenicity and *in vivo* growth assessments were performed with wild-type *Drosophila melanogaster* strain Oregon R flies. Flies were maintained on standard dextrose medium (13% dextrose, 0.74% agar, 6.12% corn meal, 3.24% yeast, 2.7% Tegosept) at 25°C and 60% humidity. All experiments were conducted on male flies 5-7d post-eclosion.

The microbial strains used in this study included *S. borellii*-isolated cultures of *Serratia marcescens* (red), *S. marcescens* (white), *S. marcescens* (purple), *Achromobacter xylosoxidans*, *Chryseobacterium species*, *Ochrobactrum anthropi*, *Beauveria bassiana*. *Serratia marcescens* strain DB1140, a non-motile mutant derived from non-pathogenic variant of parent DB10/DB11 strains (Flyg and Xanthopoulos 1983; Kurz et al. 2003; Nehme et al. 2007) and *S. marcescens* strain ATCC13880, a natural strain derived from a pond, were also used in our experiments to compare the virulence. All the bacterial cultures were grown overnight in sterile LB broth, incubated at 28°C).

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The cultures were measured for optical density (OD) at 600 nanometers, and diluted to the desired OD_{600} in sterile phosphate buffer solution (PBS). For each bacterial strain, the OD_{600} that contained 1,000 colony forming units (CFUs) in 50 nanoliters was determined experimentally by injecting 50 nl of solutions with different OD_{600} into flies and homogenization and plating the homogenate in LB plates for overnight incubation at 28°C. The experimentally determined OD_{600} corresponding to ~1,000 CFUs were as follows: *S. marcescens* (red), 0.018; *S. marcescens* (white), 0.011; *S. marcescens* (purple), 0.02; *Achromobacter xylosoxidans*, 0.05; *Chryseobacterium species*, 0.015; *Ochrobactrum anthropi*, 0.015; *S. marcescens* strain DB1140, 0.01; and *S. marcescens* strain ATCC13880, 0.015. Dosages of bacterial concentration were determined either by serially diluting or centrifugally concentrating at 12,000 g for 2 minutes. *Tsukamurella tryosinosolvens* pathogenicity assessment was not performed due to its aggregated growth in liquid culture. The only fungus isolated from *S. borellii*, *B. bassiana*, cultured in malt agar, was obtained by Stajich lab at the University of California Riverside. *B. bassiana* spores clustered in sporangia were separated using a sonicator (Qsonica Sonicators, Qsonica, LLC., Newtown, Connecticut, USA). We sonicated clumped spores at 40% amplitude, 01 pulse and 30 sec time and spore concentration was determined using hemocytometer counts. A final concentration of 2 x 10^8 spores/ml was made to obtain ~10^4 spores/50 nl injection. Serial dilutions were used to obtain reduced spore dosages.

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Injections of varying microbial doses and a PBS control into *D. melanogaster* were performed in 5-7d post-eclosion male flies as previously described (Louie et al. 2016). Flies were anesthetized with CO_2 not longer than 10 minutes and each fly received a total volume of 50 nl using individually calibrated pulled glass needles attached to a MINJ-FLY high-speed pneumatic injector (Tritech Research, CA). Flies were injected in the anterior abdomen, close to the junction with the thorax and just ventral to the junction between the ventral and dorsal cuticles. A 50 nl volume was calibrated by measuring the diameter of the ejected droplet into the immersion oil using a micrometer attached to an ocular lens. Injected flies were transferred into a fresh vial containing 20 flies, with three replications and maintained at 25°C and 60% humidity on a 12h:12h light dark cycle. Flies were placed into new vials of food every 6-7 days. Bacterial and fungal loads after each injection experiment were verified for all strains by immediately homogenizing and plating in appropriate media plates followed by overnight incubation at 28°C and 48h incubation at 30°C, respectively.

All experiments were repeated at least three times. For survival analysis, a minimum of 60 individual flies were injected for each dose of each microbe tested. Fly survival was checked every or every other day until all experimental flies died. Survival assay (dose response curve) data were graphed and analyzed using GraphPad Prism (GraphPad Software, http://www.graphpad.com).

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2.3. In vivo *growth assessment*

To determine CFUs in infected flies, each fly was homogenized in 200 µl of PBS, diluted serially, and spotted 50 µl onto LB plates supplemented with 0.1% sodium pyruvate, as previously described (Louie et al. 2016). Plates were kept overnight at 28°C and total CFUs were determined. For each *in vivo* growth assay, we injected and homogenized at least 10 flies, for each, dose and each time point for each microbe tested. Time points that are seemingly missing from our figures are due to a lack of live adult flies at particular time points and particular starting innocula. Homogenized *B. bassiana* spores were grown on PDA plates and incubated at 30°C. Germinating spores were counted at 48h and determined per dose each time point.

All CFU experiments were repeated at least three times. For the *in vivo* growth assay, a minimum of 90 flies were injected for each dose each experiment. *In vivo* growth data were graphed and analyzed using GraphPad Prism (GraphPad Software, http://www.graphpad.com).

2.4 *Disease tolerance curves*

We measured disease tolerance curves (Fig. 4) for a subset of the cricket-associated microbes using previously described methods (Dillman and Schneider 2015; Louie et al. 2016). Disease tolerance was determined by plotting host health versus microbial growth. We determined the median time to death (MTD) for each inoculum of each microbe and used this as a measure of health. For microbial growth we used the bacterial CFUs at 6h post-infection and the number

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of individual hyphal growth points of *B. bassiana* at 48h post-infection. Plotting microbe load against MTD produced curves that were fit by four-parameter logistic sigmoid models (R-square $\geq$ 0.82) (Fig. 4). For DB1140, we used a manual method to calculate EC_{50} as previously described (Stephan 1977).

3. Results

3.1. *Microbes associated with* Scapteriscus borellii *mole crickets*

We isolated and identified the culturable aerobic or facultatively aerobic microbes associated with diseased (non-feeding, reduced mobility, and decreased response to touch stimuli) and dead mole crickets collected from the Rio Hondo Golf Club in Downey, CA (Dillman et al. 2014). Seven aerobic or facultatively aerobic bacterial strains and one spore (conidia) forming fungal strain were obtained from the thorax of dead and diseased *S. borellii* (Fig. 1 and Table 1). Bacterial and fungal isolates were identified using morphological features and rRNA gene sequence analysis (16S and 18S respectively). The bacterial isolates were differentiated and identified as *Serratia marcescens* (red), *S. marcescens* (white), *S. marcescens* (purple), *Chryseobacterium sp.*, *Achromobacter xylosoxidans*, *Ochrobactrum anthropi* (resistant to carbenicillin and kanamycin), and *Tsukamurella tryosinosolvens*. The only fungus recovered was the *Beauveria bassiana* (Ascomycota; Hypocreales), although a green spore forming fungus was also observed but could not be identified due to difficulties in obtaining pure culture (data not shown). Plates with bacterial colonies and fungal sporulation (germination) were obtained by picking distinct colonies from initial

plates and then streaking for pure culture (Fig. S1). We differentiated between dead crickets and diseased crickets and microbes collected from each. The prevalence of each microbe found in dead or diseased mole crickets is shown in Table 1. We identified three distinct strains of *Serratia* with the purple (amaranth red), red (often produced a small number of non-pigmented colonies that later changed to red), and white colonies having nearly identical 16S sequences (99%).

3.2. *Virulence of microbes associated with* S. borellii *mole crickets*

Pathogencity of six of the seven bacterial strains and one fungal strain isolated from *S. borellii* was assessed individually using the model host *D. melanogaster*. *Drosophila* is a powerful model host for studying host-pathogen interactions and for revealing mechanistic details of bacterial pathogenesis (Apidianakis and Rahme 2009; Buchon et al. 2014; Louie et al. 2016). We did not assess the virulence of *T. tryosinosolvens* due to technical challenges of separating bacterial aggregates and accurately measuring inoculation inoculative doses. The pathogenicity of each microbe was tested by injecting a series of doses of a strain into adult flies to determine dose-response curves. We compared the virulence of the six bacterial strains collected from mole crickets with a mutant strain of *S. marcescens* DB1140, originally derived from strain Db10 (Flyg and Xanthopoulos 1983), that has attenuated virulence and a *S. marcescens* strain collected from pond water (ATCC 13880) (Daligault et al. 2014), not known to be associated with insects. We will refer to this pond strain

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346 as *S. marcescens* (pond) throughout. We found that five of the six bacterial
347 strains associated with mole crickets, *S. marcescens* (red), *S. marcescens*
348 (white), *S. marcescens* (purple), *Chryseobacterium sp.,* and *A. xylosoxidans*,
349 were highly pathogenic to flies compared to *S. marcescens* DB1140 and *S.*
350 *marcescens* (pond), for every dose tested (Fig. 2). The three cricket-associated
351 strains of *S. marcescens* killed all injected flies, even at the lowest initial dose
352 tested of ~10 CFUs, within 24h of infection (Fig. 2A-C). The highest dose
353 administered for all bacterial strains was ~10^5 CFUs, and this dose killed all flies
354 within 24h except for flies injected with *O. anthropi*. We found that of the mole
355 cricket-associated bacteria we tested, only *O. anthropi* was less virulent than *S.*
356 *marcescens* DB1140 and *S. marcescens* (pond); *O. anthropi* was not pathogenic,
357 and flies injected with $\leq$ 1,000 CFUs of *O. anthropic* had similar mortality rates as
358 those injected with PBS (Fig. 2F). We also found that *S. marcescens* DB1140
359 was more virulent (induced higher mortality) than *S. marcescens* (pond) for each
360 dose administered (Fig. 2G-H). Similarly, *B. bassiana*, the only mole cricket-
361 associated fungus we identified and tested, showed strong pathogenicity against
362 *D. melanogaster* and all the injected flies died faster than PBS controls, even
363 those flies injected with only ~10 spores. 10^4 spores was a highly toxic dose and
364 killed all injected flies within 5d (days) (Fig. 2I).
365 The average MTD for uninfected and PBS-injected Oregon R strain flies
366 (n=1620) was 33d and 30d, respectively. The MTD for each dose of the nine
367 microbes we injected into adult flies is summarized in Figure 2 and Table 2.
368

3.3. In vivo *growth assessment*

We studied the *in vivo* growth behavior of all microbial strains tested for pathogenicity. We found that red, white and purple strains of *Serratia, Chryseobacterium sp.*, and *A. xylosoxidans* had significantly higher growth as indicated by measurable CFUs for each time point assayed than *S. marcescens* DB1140 or *S. marcescens* (pond) (Fig. 3). The *in vivo* growth of *S. marcescens* red and white were similar to each other, reaching ≥ 1 million CFUs after 12h when only ~10 CFUs were initially injected (Fig. 3A-B and Fig. S2A-B). For *S. marcescens* (purple) and *Chryseobacterium sp.*, we performed only 6h *in vivo* growth assays, while for *A. xylosoxidans* we performed 6h and 24h growth counts (Fig. S2C). *In vivo* growth data revealed that *S. marcescens* (purple), *Chryseobacterium sp*., and *A. xylosoxidans* grew slower by 6h postinfection compared to red and white *S. marcescens*, but faster than *S. marcescens* DB1140 or *S. marcescens* (pond) (Fig. 3).

We found that a large number of cells of *O. anthropi, S. marcescens* DB1140 and *S. marcescens* (pond) can be tolerated by *D. melanogaster*. Only ~10^5 CFUs of *S. marcescens* DB1140 and *S. marcescens* (pond) were able to kill injected flies overnight whereas the same dose of *O. anthropi* was unable to kill even 50% of the injected flies until 9 DPI (Fig. 2F). We observed that *O. anthropi* grew slowly at first, going from 14 CFUs to only 71 CFUs by 6h postinfection, and by 24h postinfection we noticed a reduction in CFUs (from 71 CFUs to 43 CFUs), which may be a result of resistance from the fly or the conditions in the hemocoel (Fig. S2F). We found that *S. marcescens* DB1140 and *S. marcescens* (pond) hit

392 a plateau in growth by 6h postinfection and were subsequently reduced in CFUs
393 but never cleared from the flies (Fig. 2G-H and Fig. S2G-H). When measuring
394 the *in vivo* growth of *B. bassiana*, we found little to no growth until 3 DPI for each
395 dose (Fig. 3I). However, after this initial lag, we observed significant *B. bassiana*
396 growth by 6 and 7 DPI, for flies injected with ~10 and ~100 spores (Fig. 3I). It is
397 likely that at least part of the lag in *B. bassiana* growth we observed is an artifact
398 of how we measured fungal growth, which depended on germination of spores or
399 hyphal growth on plates, and the time required for fungal germination and
400 sporulation.
401
402 3.4. *Disease tolerance curves*
403 We measured disease tolerance curves (Fig. 4) for some of the cricket-
404 associated microbes using previously described methods (Dillman and Schneider
405 2015; Louie et al. 2016). For microbial growth we used the number of bacterial
406 CFUs present at 6h postinfection and the number of individual hyphal growth
407 points of *B. bassiana* at 24h postinfection. Plotting microbe load against MTD
408 produced curves that were fit by four-parameter logistic sigmoid models ($r^2 \geq$
409 0.82) (Fig. S3). By fitting the data with a sigmoid model, we have four parameters
410 that can be evaluated: vigor, slope, EC_{50}, and disease severity (Louie et al.
411 2016). Because we used the same fly strain for all of the experiments (Oregon
412 R), the health of uninfected animals or "vigor" is the same in all our disease
413 tolerance curves (Fig. 4). The asymptotic tail end of the sigmoid models at high
414 microbe loads illustrates the maximum death rate or disease severity, which we

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found to be similar for all microbes tested except *O. anthropi*, which had a lower maximum death rate even at the highest dose tested (Fig. 4B). The steepest slopes we measured in these disease tolerance curves were for *S. marcescens* (red) and *B. bassiana* (Fig. 4) (Table 3). The EC_{50} is the number of microbes present at a given time point that caused a 50% change in MTD. For the bacterial pathogens, we used 6h as our time point for measuring growth as that was the longest time point we could use and still have living infected flies to measure for each pathogen and each initial dose (Table 3). For *B. bassiana* we measured the growth at 48h postinfection since no growth was observed by 6h. The more pathogenic microbes such as *S. marcescens* (red) and *B. bassiana* have much lower EC_{50}s than the less pathogenic microbes (Table 3). The extremely low EC_{50} we measured for *B. bassiana* is likely an artifact of the way we measured fungal growth, which depended on germination of spores or hyphal growth on plates (Fig. 4E). MTD was about 15h for the lowest dose of *S. marcescens* (red), indicating that *D. melanogaster* has little to no tolerance for this bacterium compared to the infection by a corresponding dose of mutant strain *S. marcescens* DB1140 (MTD: 21d) and the pond strain of *S. marcescens* (MTD: 32d) (Fig. 4) (Table 2). *D. melanogaster* demonstrated higher tolerance to *S. marcescens* (pond) (MTD: 17d) even at a high dose (10^4 CFUs) (Fig. 4D), compared to a corresponding dose of *S. marcescens* DB1140 (MTD: 9.5d) (Fig. 4C) (Table 2). These bacteria grew to similar levels 1, 2, and 7 DPI (Fig. S2G-H). We observed the highest tolerance in *D. melanogaster* for *O. anthropi,*

even at high doses (10^5 CFUs; MTD: 9d) compared to similar doses of both *S. marcescens* DB1140 and *S. marcescens* (pond) (MTD: 1d) (Fig. 4).

4. Discussion

The southern mole cricket, *Scapteriscus borellii*, is an invasive turfgrass pest that seems to be spreading (Dillman et al. 2014; García 2006; Nickle and Castner 1984; Walker and Nickle 1981), despite the publication and use of successful management strategies in heavily infested areas. Although many biological control studies have been conducted on *Scapteriscus* mole crickets (Mhina et al. 2016), there has been no study on the microbial associates of these crickets. Here we have isolated and identified microbes associated with *S. borellii* individuals caught in the field, and have assessed their pathogenicity and *in vivo* growth in the fruit fly, *D. melanogaster*. We found that many of the easily culturable microbes associated with *S. borellii* are highly pathogenic to fruit fly adults, killing them within 3d, even at low initial inocula.

We isolated three strains of *S. marcescens* from both dead and diseased *S. borellii* with different and distinct colony pigmentation. *S. marcescens* strains are members of Enterobacteriaceae and cause diseases in plants and in a wide range of invertebrate and vertebrate hosts (Grimont and Grimont 2006). All three *S. borellii*-associated strains of *S. marcescens* we have identified were highly toxic to *D. melanogaster* adults, killing infected flies in less than 24h, even at low doses (~10 CFUs). These strains demonstrated rapid *in vivo* growth, going from 10 CFUs to over 1 million CFUs within 12h. These results suggest that mole

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cricket-associated strains of *S. marcescens* may avoid, suppress, or even lack sensitivity to the fly immune response, though more strains need to be tested. Previous studies have demonstrated that wild-type *S. marcescens* suppresses the immune response of *D. melanogaster* (Nehme et al. 2007) and our virulence data support this notion. It has also been reported that the mortality rate between wild-type flies and *toll* and *imd* mutant flies is similar in *S. marcescens* infections, and that *S. marcescens* can resist host-produced AMPs because of the presence of LPS-O-antigen (Nehme et al. 2007). *Serratia marcescens* DB1140 is reported to have pleiotropic defects; it is partially deficient in protease activity and it produces a truncated lipopolysaccharide (LPS) lacking the O-antigen, making the strain sensitive to the *imd*-dependent immune response and therefore has reduced virulence compared to wild type (Flyg and Xanthopoulos 1983; Kurz et al. 2003; Nehme et al. 2007). Our data revealed that *S. marcescens* DB1140 and *S. marcescens* (pond) (ATCC 13880) were less virulent in *D. melanogaster* than the cricket-associated *Serratia* strains. When compared to each other, *S. marcescens* (pond) had lower virulence than *S. marcescens* DB1140 at every dose we tested. Both of these were still significantly more virulent than PBS controls. These data suggest that a deeper comparative investigation of virulence factors in the pond strain and other strains that likely do not interact with insects with virulent strains isolated from animal hosts may increase our understanding of *S. marcescens* virulence in insects.

In addition to *S. marcescens*, we isolated a yellow-pigmented colony-forming member of Flavobacteriaceae, *Chryseobacterium sp.*, and a non-

pigmented colony-forming member of Alcaligenaceae, *A. xylosoxidans*. Both are Gram-negative, opportunistic clinical pathogens (Coward et al. 2016; Kirby et al. 2004; Lin et al. 2010). We found that both bacteria are highly pathogenic to *D. melanogaster* when injected into the hemocoel. However, their growth at 6h was slower than mole cricket associated *Serratia* strains, suggesting that these two bacterial strains are either slower growing in general or that they are more sensitive to the fly immune response.

We also isolated a non-pigmented Gram-negative opportunistic and nosocomial human pathogen, *O. anthropi*, a member of Brucellaceae that colonizes a wide range of invertebrate and vertebrate organisms (Romano et al. 2009). Our results, based on fly survival, indicated that *D. melanogaster* was able to tolerate a large number of *O. anthropi* compared to other bacteria isolated from *S. borellii*. *In vivo* growth data revealed that after 14 DPI, there were still a substantial number of bacteria inside the fly, indicating that the fly has a high tolerance for this bacterium and/or that this bacterium has low virulence against *D. melanogaster* compared to the other microbes we tested.

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We isolated *B. bassiana*, a member of Cordycipitaceae, from field-caught *S. borellii* mole crickets. This is a common entomopathogenic fungus and we isolated it from more than 30% *S. borellii* that died after being collected from the field. We found that *B. bassiana* caused 50% mortality of fly populations within 10d in flies injected with just ~10 spores. In our experiments we treated flies injected with *B. bassiana* similar to flies injected with bacteria. To measure growth we ground up the flies and plated the homogenate on potato dextrose

agar (PDA) plates. We measured microbial growth by counting the number of independent regions of hyphal growth on these PDA plates, similar to how colony-forming units of bacteria are counted. The lack of observed *B. bassiana* growth in our assays may also be due to longer spore germination and sporulation time, however, there was a rapid increase in fungal growth at 6 and 7 DPI. Our data suggested that once this fungus enters the hemocoel, even at low doses, the fly is unable to successfully resist this pathogen. Flies respond to fungal infection by activating the Toll pathway and triggering the activation of AMPs, particularly Drosomycin (Drs) and Metchnikowin (Lemaitre and Hoffmann 2007). It is not yet known how this fungus kills the fly, but many fungal pathogens produce mycotoxins and kill their hosts through invasive growth and by depleting host nutrients (Samuels et al. 1988).

Because we had measured host health and microbial growth in flies infected with the mole cricket-associated microbes we have isolated, we used this information and plotted disease tolerance curves for some of these microbes (Fig. 4 and S3). These curves helped us understand the infection dynamics of these microbes in flies and disease tolerance curves in general allow researchers to study the relative contributions of resistance and tolerance to immune defense separately (Howick and Lazzaro 2017; Louie et al. 2016). The disease tolerance curves revealed the heath or fitness of a population at a given pathogen burden. In the case of the highly virulent pathogens, we found that any amount of pathogen dramatically reduced the health of the infected flies, while for less virulent microbes, low doses had almost no effect on host health. Although

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disease tolerance data are usually fitted with linear models (Ayres and Schneider 2008; Dillman and Schneider 2015; Råberg et al. 2007), we found that these data were best fit with sigmoid models, which provided four parameters for comparison (Louie et al. 2016). The EC_{50} of each system seems to be a particularly useful piece of data, as it reveals the number of microbes (present at some time point) that cause a 50% change in host health. In our case it revealed the number of bacterial pathogens present after 6h postinfection, and the amount of fungus 24h postinfection that lead to a 50% change in host health. For host health we were measuring median time to death (MTD), but any measure of health could be used. While relatively few studies currently plot disease tolerance curves, they reveal information about the host health and microbe interaction that are not apparent using microbial growth and host health measures alone. Therefore, it seems practical that more researchers would adopt this technique and measure disease tolerance, especially since such plots provide additional parameters that have not been well-studied in microbial pathogenesis such as disease severity and EC_{50} that may be useful (Louie et al. 2016).

Although effective strategies of biological control have been developed and used against *S. borellii* mole crickets, previous data suggests that these crickets are quite resilient to some infections (Dillman et al. 2012; Nguyen and Smart 1991). For example, *St. scapterisci,* a natural parasite of *S. borellii* mole crickets has limited effectiveness against these crickets. One study reported that only 25% of *S. borellii* mole crickets died when exposed to 100 *St. scapterisci* infective juveniles (Dillman et al. 2012), while another study reported 75%

mortality when the crickets were exposed to 800 *St. scapterisci* infective juveniles (Nguyen and Smart 1991). The generalist insect parasite *St. carpocapsae* had even lower virulence against *S. borellii*, where only 15% of mole cricket adults died when exposed to 100 *St. carpocapsae* infective juveniles (Dillman et al. 2012). These studies suggest that *S. borellii* has evolved strategies that allow it to avoid or resist infection. Mole crickets inhabit diverse niches and interact with numerous microbes; they are largely subterranean during the day and can fly at night. Their interaction and/or association with highly pathogenic microbes in the soil may contribute to the evolution of an especially robust immune response against soil-dwelling parasites and pathogenic microbes, though this remains to be tested.

Here we isolated and identified bacteria and fungi from the thorax of dead and diseased *Scapteriscus* mole crickets that had been caught in the field. Most of the microbes we identified were highly virulent to flies when injected into the hemolymph of fruit flies. Although we isolated and cultured seven different microbes, *S. marcescens* and *B. bassiana* were the most common and we imagine they were primarily responsible for the mortality we observed in our field-caught crickets. We speculate that the crickets' association with highly pathogenic bacteria has driven the evolution of a strong immune response.

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719 **Tables and Figures**

720 Table 1. Percentage occurrence of microbes isolated from *S. borellii*.

Species name	% occurrence in dead *S. borellii* (n=30)	% occurrence in diseased *S. borellii* (n=10)
Serratia marcescens (red)	80	80
Serratia marcescens (white)	60	80
Serratia marcescens (purple)	46	60
Achromobacter xylosoxidans	40	60
Chryseobacterium species	50	50
Ochrobactrum anthropi	43	50
Tsukamurella tryosinosolvens	27	20
Beauveria bassiana	37	30

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722 Table 2. Median time to death for number of CFUs administered for each dose (treatment).

	Median time to death (days)						
	Approximate number of CFUs administered each dose (n=180)						
Microbial strain	Control	PBS	10^1	10^2	10^3	10^4	10^5
Serratia marcescens (red)	33	29	1	1	1	1	1
Serratia marcescens (white)	34	32	1	1	1	1	1
Serratia marcescens (purple)	31	31	1	1	1	1	1
Achromobacter xylosoxidans	32	31	3	2	2	2	1

Chryseobacterium species	32	30	1	1	1	1	1
Ochrobactrum anthropi	30	32	31	30	29	24	9
Serratia marcescens DB1140	37	30	21	19	15	9.5	1
Serratia marcescens (pond)	35	30	32	29	23	17	1
Beauveria bassiana	38	33	10	8	6	3	

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728 Table 3. Parameters used to measure disease tolerance based on MTD reported in (Table 2).

Microbial strain	EC_{50}	Slope
Serratia marcescens (red)	14.97	-3.841
Serratia marcescens (white)	19.36	-0.905
Serratia marcescens (purple)	3.186	-1.143
Achromobacter xylosoxidans	12.20	-1.417
Chryseobacterium species	12.81	-1.639
Ochrobactrum anthropi	10.9×10^7	-0.283
Serratia marcescens DB1140	1043	-0.057
Serratia marcescens (pond)	77,648	-0.541
Beauveria bassiana	1.06	-3.017

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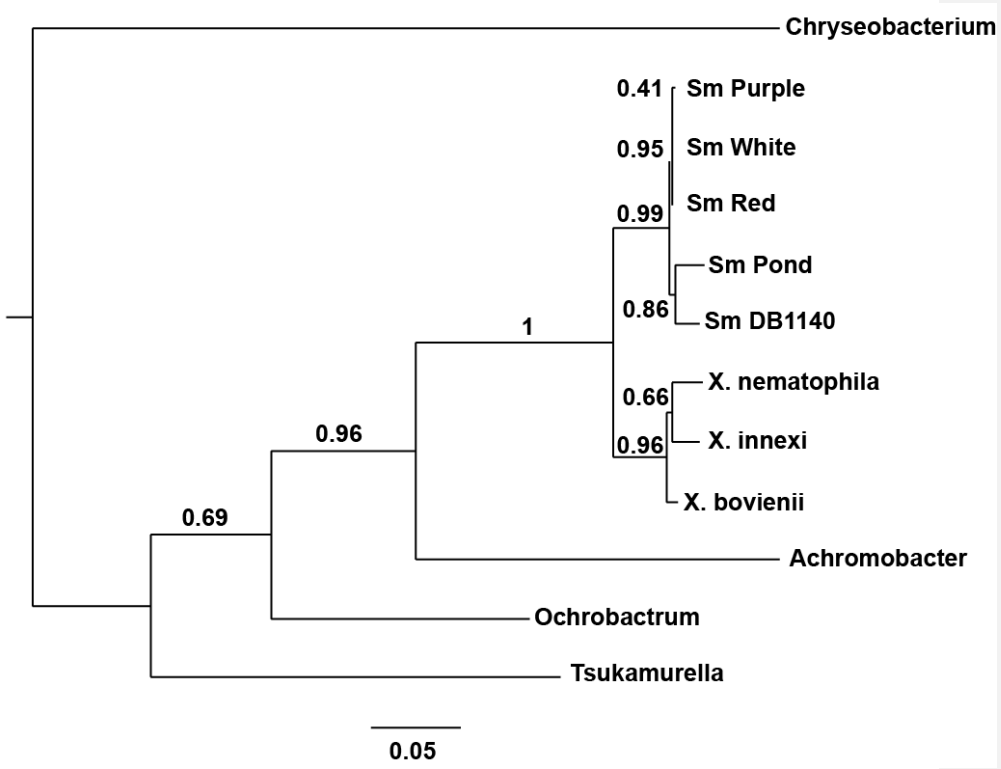


Figure 1. Phylogenetic relationships of bacteria isolated from mole crickets based on maximum likelihood analyses. Bootstrap support values indicated above the branch. Taxon labels are based on best reciprocal hits of the 16S sequence from BLAST.

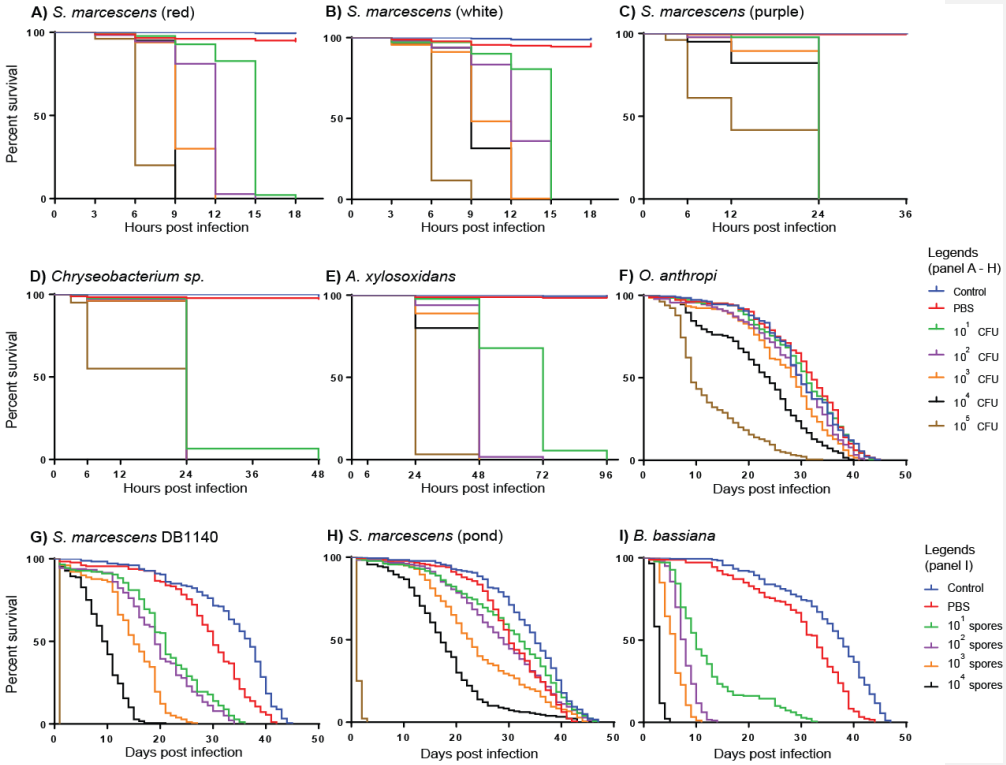
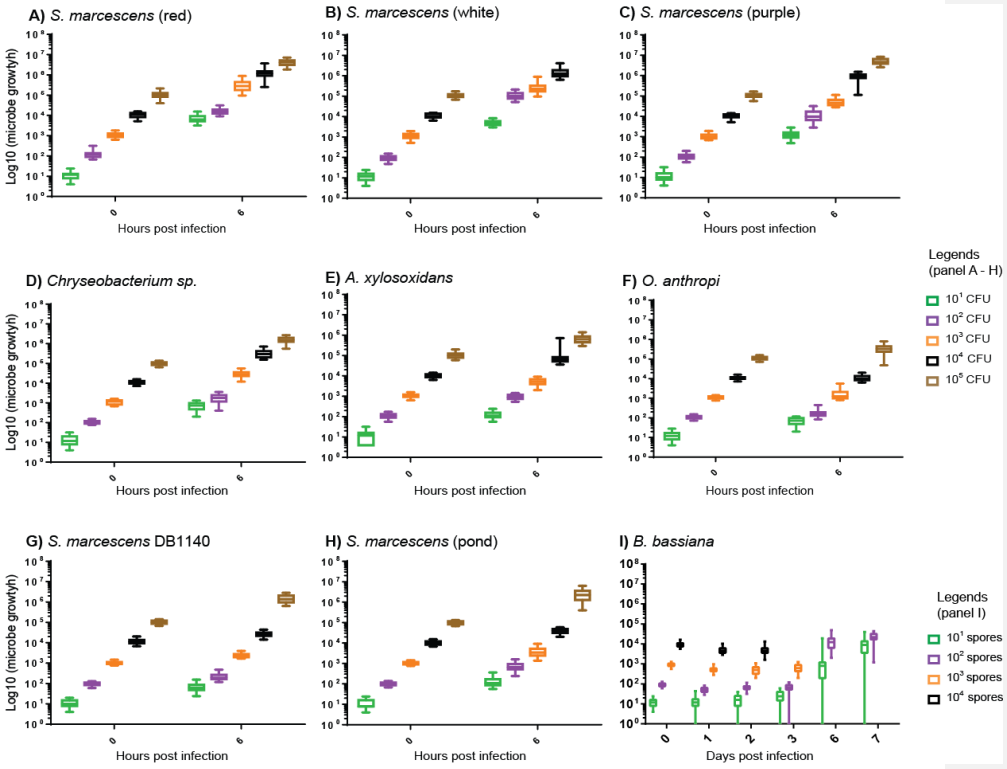


Figure 2. Survival assays. (A) *Serratia marcescens* (red). (B) *S. marcescens* (white). (C) *S. marcescens* (purple). (D) *Chryseobacterium species*. (E) *Achromobacter xylosoxidans*. (F) *Ochrobactrum anthropi*. (G) *S. marcescens* DB1140. (H) *S. marcescens* (pond). (I) *Beauveria bassiana.*



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744 **Figure 3.** *In vivo* growth assays. (A) *Serratia marcescens* (red). (B) *S.*
745 *marcescens* (white). (C) *S. marcescens* (purple). (D) *Chryseobacterium species*.
746 (E) *Achromobacter xylosoxidans*. (F) *Ochrobactrum anthropi*. (G) *S. marcescens*
747 DB1140. (H) *S. marcescens* (pond). (I) *Beauveria bassiana.*

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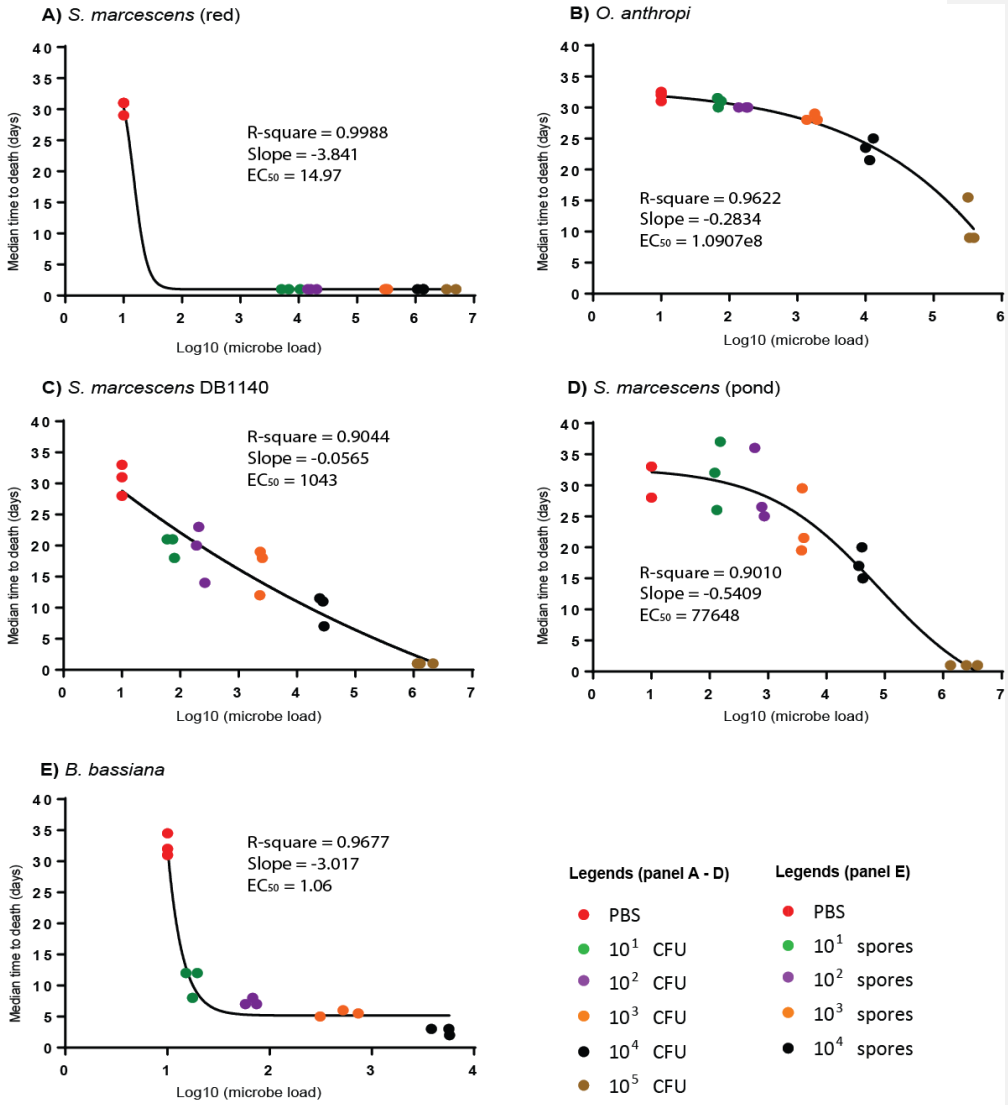

Figure 4. Tolerance curves of select microbes. These curves are fit with four parameter sigmoid models, allowing for the measure and comparison of four different parameters: vigor, slope, EC_{50}, and disease severity. The steepness of the slopes reveals the virulence of the microbes or the tolerance of the host, with steep slopes indicating higher virulence of the microbe or lower tolerance by the host than shallow slopes. (A) *Serratia marcescens* (red) disease tolerance. (B) *Ochrobactrum anthropi* disease tolerance. C) *S. marcescens* DB1140 disease

tolerance. (D) *S. marcescens* (pond) disease tolerance. (E) *Beauveria bassiana* disease tolerance.

Figure S1. Pictures of the microbial cultures isolated from *S. borellii* mole crickets. (A) *S. marcescens* (red). The whitish colonies in this image became red after a few days. (B) *S. marcescens* (white). (C) *S. marcescens* (purple). The colonies took on a purple hue after a few days. (D) *Chryseobacterium* species. (E) *Achromobacter xylosoxidans*. (F) *Ochrobactrum anthropi*. (G) *Tsukamurella tryosinosolvens*. (H) *Beauveria bassiana* after 96h. (I) *Beauveria bassiana* after 48h.

Figure S2. Growth curves of injected bacteria. These plots show bacterial growth overtime, with different initial innocula. (A) *Serratia marcescens* (red). (B) *Serratia marcescens* (white). C) *A. xylosoxidans*. (D) *Ochrobactrum anthropi*. (E) *Serratia marcescens* (DB1140). (F) *Serratia marcescens* (pond).

Figure S3. A cartoon of a disease-tolerance curve. The drawing shows the parameters used to describe a sigmoid disease-tolerance curve including vigor, slope, EC_{50}, and maximal disease severity.