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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 ≤ 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*

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

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

107 number 3144). Each individual adult was marked and kept in a container
108 fabricated from PVC pipe and polystyrene Petri dish lids (Fisher #0875712). A
109 piece of schedule 40 PVC coupling pipe 8.3 cm long with an outside diameter of
110 8.9 cm was capped on each end with a Petri dish lid, 9.1 cm x 0.9 cm (outside
111 diam x height). The top lid was ventilated by drilling 6-8 holes while an undrilled
112 lid was used as the bottom. Each container was filled with ~250 cc of clean,
113 autoclaved and lightly moistened sand. The Petri dish lids, serving as the top and
114 bottom, were secured to the PVC pipe with #64 rubber bands. Each individual
115 was fed twice a week and any old food present at the next feeding was removed
116 using sterile tweezers. Any container with dry sand was lightly moistened with tap
117 water as needed throughout the rearing process. All the *S. borellii* crickets were
118 maintained at 25°C and 60% humidity. Freshly dead and diseased *S. borellii*
119 were collected for this study.

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

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

150 Single colonies of bacteria strains were selected and grown in 5 mL liquid
151 LB overnight agitated at 200 RPM at 30°C in a shaking incubator. 2 mL of liquid
152 culture were spun down at 15,000 RPM for 30 seconds using a tabletop
153 centrifuge. Supernatant was discarded and the pellet was vigorously
154 resuspended with 500 µL of 2% Cetyltrimethylammonium bromide preheated to
155 65°C. Samples were incubated at 65°C for 15-30 minutes. Next 500 µL of 24:1
156 Chloroform:Isoamyl Alcohol was added and thoroughly mixed by inversion (>2
157 min). Samples were then spun at 7,000 g for 10 minutes in a tabletop centrifuge.

158 Upper aqueous supernatant was pipetted into fresh 1.5 mL tubes then 3M
159 Sodium Acetamide (1/10 volume) was added followed by Isopropanol (1 volume).
160 After a few gentle inversions the samples were centrifuged at 3,000 g for 2
161 minutes, then the supernatant was decanted. To wash the DNA 500 µL freshly
162 prepared cold 70% ethanol was added followed by an additional spin (3,000 g, 2
163 min), then decanted. The pellet was dried at 65°C for 2 minutes then suspended
164 in 250 µL DEPC water. 1 µL was carried forward as template for PCR using the
165 following primers to amplify 16S/18S sequences (36, 37): 16S 8F – 5'-
166 AGAGTTTGATCMTGGCTCAG-3', 16S 1492R – 5'-
167 GGTACCTTGTACGACTT-3', 18S - 5'-AAACGGCTACCACATCCAAG-3', 18S
168 - 5'-GTACAAAGGGCAGGGACGTA-3'.

169 Amplicons were sequenced with Sanger BigDye chemistry on an Applied
170 Biosystems 3730xl DNA sequencer, trimmed using Chromatogram Explorer v.
171 3.2 and species identification made by best hits to the 16S database using
172 BLASTN. All sequences were combined into a single file in FastA format and
173 uploaded to www.phylogeny.fr to perform multiple alignments, trimming, and
174 phylogenetic inference (Anisimova and Gascuel 2006; Dereeper et al. 2008;
175 Dereeper et al. 2010;). Sequences were aligned using MUSCLE (Edgar 2004;
176 v3.8.31) with default settings and trimmed using Gblocks (Castresana 2000;
177 v0.91b) with the following parameters: minimum length of a block after gap
178 removal: 10, no gap positions were allowed in the terminal positions in the
179 alignment, all segments with contiguous nonconserved positions bigger than 8
180 were rejected, minimum number of sequences for a flank position: 85%. A gene

181 tree from the 16S sequences was created by PhyML program (Guindon and
182 Gascuel 2003; v3.1/3.0 aLRT) using the following settings: Model: HKY85,
183 Statistical test: aLRT, Number of categories: 4, Gamma: 0.725, Invariable sites:
184 0.369, Remove gaps: enabled, 100 bootstraps. The tree was visualized using
185 Figtree (Rambaut and Drummond 2009; v. 1.3.1). The 16S and 18S sequences
186 | were submitted to Genbank and assigned accession numbers MF103678-
187 MF103684.

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189 2.2. Virulence of microbes associated with *S. borellii* mole crickets

190 All the pathogenicity and *in vivo* growth assessments were performed with
191 wild-type *Drosophila melanogaster* strain Oregon R flies. Flies were maintained
192 on standard dextrose medium (13% dextrose, 0.74% agar, 6.12% corn meal,
193 3.24% yeast, 2.7% Tegosept) at 25°C and 60% humidity. All experiments were
194 conducted on male flies 5-7d post-eclosion.

195 The microbial strains used in this study included *S. borellii*-isolated
196 cultures of *Serratia marcescens* (red), *S. marcescens* (white), *S. marcescens*
197 (purple), *Achromobacter xylosoxidans*, *Chryseobacterium species*,
198 *Ochrobactrum anthropi*, *Beauveria bassiana*. *Serratia marcescens* strain
199 DB1140, a non-motile mutant derived from non-pathogenic variant of parent
200 DB10/DB11 strains (Flyg and Xanthopoulos 1983; Kurz et al. 2003; Nehme et al.
201 2007) and *S. marcescens* strain ATCC13880, a natural strain derived from a
202 pond, were also used in our experiments to compare the virulence. All the
203 | bacterial cultures were grown overnight in sterile LB broth, incubated at 28°C).

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210 The cultures were measured for optical density (OD) at 600 nanometers, and
211 diluted to the desired OD₆₀₀ in sterile phosphate buffer solution (PBS). For each
212 bacterial strain, the OD₆₀₀ that contained 1,000 colony forming units (CFUs) in 50
213 nanoliters was determined experimentally by injecting 50 nl of solutions with
214 different OD₆₀₀ into flies and homogenization, and plating the homogenate in LB
215 plates for overnight incubation at 28°C. The experimentally determined OD₆₀₀
216 corresponding to ~1,000 CFUs were as follows: *S. marcescens* (red), 0.018; *S.*
217 *marcescens* (white), 0.011; *S. marcescens* (purple), 0.02; *Achromobacter*
218 *xylosoxidans*, 0.05; *Chryseobacterium species*, 0.015; *Ochrobactrum anthropi*,
219 0.015; *S. marcescens* strain DB1140, 0.01; and *S. marcescens* strain
220 ATCC13880, 0.015. Dosages of bacterial concentration were determined either
221 by serially diluting or centrifugally concentrating at 12,000 g for 2 minutes.
222 *Tsukamurella tryosinosolvens* pathogenicity assessment was not performed due
223 to its aggregated growth in liquid culture. The only fungus isolated from *S.*
224 *borellii*, *B. bassiana*, cultured in malt agar, was obtained by Stajich lab at the
225 University of California Riverside. *B. bassiana* spores clustered in sporangia
226 were separated using a sonicator (Qsonica Sonicators, Qsonica, LLC., Newtown,
227 Connecticut, USA). We sonicated clumped spores at 40% amplitude, 01 pulse
228 and 30 sec time and spore concentration was determined using hemocytometer
229 counts. A final concentration of 2 x 10⁸ spores/ml was made to obtain ~10⁴
230 spores/50 nl injection. Serial dilutions were used to obtain reduced spore
231 dosages.

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242 Injections of varying microbial doses and a PBS control into *D.*
243 *melanogaster* were performed in 5-7d post-eclosion male flies as previously
244 described (Louie et al. 2016). Flies were anesthetized with CO₂ not longer than
245 10 minutes and each fly received a total volume of 50 nl using individually
246 calibrated pulled glass needles attached to a MINJ-FLY high-speed pneumatic
247 injector (Tritech Research, CA). Flies were injected in the anterior abdomen,
248 close to the junction with the thorax and just ventral to the junction between the
249 ventral and dorsal cuticles. A 50 nl volume was calibrated by measuring the
250 diameter of the ejected droplet into the immersion oil using a micrometer
251 attached to an ocular lens. Injected flies were transferred into a fresh vial
252 containing 20 flies, with three replications and maintained at 25°C and 60%
253 humidity on a 12h:12h light dark cycle. Flies were placed into new vials of food
254 every 6-7 days. Bacterial and fungal loads after each injection experiment were
255 verified for all strains by immediately homogenizing and plating in appropriate
256 media plates followed by overnight incubation at 28°C and 48h incubation at
257 30°C, respectively.

258 All experiments were repeated at least three times. For survival analysis, a
259 minimum of 60 individual flies were injected for each dose of each microbe
260 tested. Fly survival was checked every or every other day until all experimental
261 flies died. Survival assay (dose response curve) data were graphed and
262 analyzed using GraphPad Prism (GraphPad Software,
263 <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 inocula. 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 ($R\text{-square} \geq 0.82$) (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

Pathogenicity 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 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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as *S. marcescens* (pond) throughout. We found that five of the six bacterial strains associated with mole crickets, *S. marcescens* (red), *S. marcescens* (white), *S. marcescens* (purple), *Chryseobacterium* sp., and *A. xylosoxidans*, were highly pathogenic to flies compared to *S. marcescens* DB1140 and *S. marcescens* (pond), for every dose tested (Fig. 2). The three cricket-associated strains of *S. marcescens* killed all injected flies, even at the lowest initial dose tested of ~10 CFUs, within 24h of infection (Fig. 2A-C). The highest dose administered for all bacterial strains was $\sim 10^5$ CFUs, and this dose killed all flies within 24h except for flies injected with *O. anthropi*. We found that of the mole cricket-associated bacteria we tested, only *O. anthropi* was less virulent than *S. marcescens* DB1140 and *S. marcescens* (pond); *O. anthropi* was not pathogenic, and flies injected with $\leq 1,000$ CFUs of *O. anthropic* had similar mortality rates as those injected with PBS (Fig. 2F). We also found that *S. marcescens* DB1140 was more virulent (induced higher mortality) than *S. marcescens* (pond) for each dose administered (Fig. 2G-H). Similarly, *B. bassiana*, the only mole cricket-associated fungus we identified and tested, showed strong pathogenicity against *D. melanogaster* and all the injected flies died faster than PBS controls, even those flies injected with only ~10 spores. 10^4 spores was a highly toxic dose and killed all injected flies within 5d (days) (Fig. 2I).

The average MTD for uninfected and PBS-injected Oregon R strain flies (n=1620) was 33d and 30d, respectively. The MTD for each dose of the nine microbes we injected into adult flies is summarized in Figure 2 and Table 2.

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 $\sim 10^5$ 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

a plateau in growth by 6h postinfection and were subsequently reduced in CFUs but never cleared from the flies (Fig. 2G-H and Fig. S2G-H). When measuring the *in vivo* growth of *B. bassiana*, we found little to no growth until 3 DPI for each dose (Fig. 3I). However, after this initial lag, we observed significant *B. bassiana* growth by 6 and 7 DPI, for flies injected with ~10 and ~100 spores (Fig. 3I). It is likely that at least part of the lag in *B. bassiana* growth we observed is an artifact of how we measured fungal growth, which depended on germination of spores or hyphal growth on plates, and the time required for fungal germination and sporulation.

3.4. Disease tolerance curves

We measured disease tolerance curves (Fig. 4) for some of the cricket-associated microbes using previously described methods (Dillman and Schneider 2015; Louie et al. 2016). For microbial growth we used the number of bacterial CFUs present at 6h postinfection and the number of individual hyphal growth points of *B. bassiana* at 24h postinfection. Plotting microbe load against MTD produced curves that were fit by four-parameter logistic sigmoid models ($r^2 \geq 0.82$) (Fig. S3). By fitting the data with a sigmoid model, we have four parameters that can be evaluated: vigor, slope, EC_{50} , and disease severity (Louie et al. 2016). Because we used the same fly strain for all of the experiments (Oregon R), the health of uninfected animals or "vigor" is the same in all our disease tolerance curves (Fig. 4). The asymptotic tail end of the sigmoid models at high microbe loads illustrates the maximum death rate or disease severity, which we

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417 found to be similar for all microbes tested except *O. anthropi*, which had a lower
418 maximum death rate even at the highest dose tested (Fig. 4B). The steepest
419 slopes we measured in these disease tolerance curves were for *S. marcescens*
420 (red) and *B. bassiana* (Fig. 4) (Table 3). The EC₅₀ is the number of microbes
421 present at a given time point that caused a 50% change in MTD. For the bacterial
422 pathogens, we used 6h as our time point for measuring growth as that was the
423 longest time point we could use and still have living infected flies to measure for
424 each pathogen and each initial dose (Table 3). For *B. bassiana* we measured the
425 growth at 48h postinfection since no growth was observed by 6h. The more
426 pathogenic microbes such as *S. marcescens* (red) and *B. bassiana* have much
427 lower EC₅₀s than the less pathogenic microbes (Table 3). The extremely low
428 EC₅₀ we measured for *B. bassiana* is likely an artifact of the way we measured
429 fungal growth, which depended on germination of spores or hyphal growth on
430 plates (Fig. 4E). MTD was about 15h for the lowest dose of *S. marcescens* (red),
431 indicating that *D. melanogaster* has little to no tolerance for this bacterium
432 compared to the infection by a corresponding dose of mutant strain *S.*
433 *marcescens* DB1140 (MTD: 21d) and the pond strain of *S. marcescens* (MTD:
434 32d) (Fig. 4) (Table 2). *D. melanogaster* demonstrated higher tolerance to *S.*
435 *marcescens* (pond) (MTD: 17d) even at a high dose (10⁴ CFUs) (Fig. 4D),
436 compared to a corresponding dose of *S. marcescens* DB1140 (MTD: 9.5d)
437 (Fig. 4C) (Table 2). These bacteria grew to similar levels 1, 2, and 7 DPI (Fig.
438 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.

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

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510 agar (PDA) plates. We measured microbial growth by counting the number of
511 independent regions of hyphal growth on these PDA plates, similar to how
512 colony-forming units of bacteria are counted. The lack of observed *B. bassiana*
513 growth in our assays may also be due to longer spore germination and
514 sporulation time, however, there was a rapid increase in fungal growth at 6 and 7
515 DPI. Our data suggested that once this fungus enters the hemocoel, even at low
516 doses, the fly is unable to successfully resist this pathogen. Flies respond to
517 fungal infection by activating the Toll pathway and triggering the activation of
518 AMPs, particularly Drosomycin (Drs) and Metchnikowin (Lemaitre and Hoffmann
519 2007). It is not yet known how this fungus kills the fly, but many fungal pathogens
520 produce mycotoxins and kill their hosts through invasive growth and by depleting
521 host nutrients (Samuels et al. 1988).

522 Because we had measured host health and microbial growth in flies
523 infected with the mole cricket-associated microbes we have isolated, we used
524 this information and plotted disease tolerance curves for some of these microbes
525 (Fig. 4 and S3). These curves helped us understand the infection dynamics of
526 these microbes in flies and disease tolerance curves in general allow researchers
527 to study the relative contributions of resistance and tolerance to immune defense
528 separately (Howick and Lazzaro 2017; Louie et al. 2016). The disease tolerance
529 curves revealed the health or fitness of a population at a given pathogen burden.
530 In the case of the highly virulent pathogens, we found that any amount of
531 pathogen dramatically reduced the health of the infected flies, while for less
532 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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References

- Akhurst RJ, Dunphy GB (1993) Tripartite interactions between symbiotically associated entomopathogenic bacteria, nematodes, and their insect hosts. In: Beckage NE, Thompson SN, Federici B (eds) *Parasites and Pathogens of Insects*. vol 2. Academic Press, New York, NY, p 1-23
- Anisimova M, Gascuel O (2006) Approximate likelihood-ratio test for branches: A fast, accurate, and powerful alternative. *Systematic Biology* 55(4):539-552 doi:10.1080/10635150600755453
- Apidianakis Y, Rahme LG (2009) *Drosophila melanogaster* as a model host for studying *Pseudomonas aeruginosa* infection. *Nature protocols* 4(9):1285-94 doi:10.1038/nprot.2009.124
- Arakelian G (2008) Southern mole cricket (*Scapteriscus borellii*). County of Los Angeles Weights and Measures Department. https://www.cdfa.ca.gov/plant/PPD/PDF/Scapteriscus_borellii.pdf
- Ayres JS, Schneider DS (2008) A signaling protease required for melanization in *Drosophila* affects resistance and tolerance of infections. *PLoS Biol* 6(12):2764-73 doi:10.1371/journal.pbio.0060305
- Buchon N, Silverman N, Cherry S (2014) Immunity in *Drosophila melanogaster*--from microbial recognition to whole-organism physiology. *Nature reviews Immunology* 14(12):796-810 doi:10.1038/nri3763
- Castresana J (2000) Selection of conserved blocks from multiple alignments for their use in phylogenetic analysis. *Molecular Biology and Evolution* 17(4):540-552
- Coward A, Kenna DTD, Perry C, Martin K, Doumith M, Turton JF (2016) Use of *nrdA* gene sequence clustering to estimate the prevalence of different *Achromobacter* species among Cystic Fibrosis patients in the UK. *J Cyst Fibros* 15(4):479-485 doi:10.1016/j.jcf.2015.09.005
- Daligault HE, et al. (2014) Genome assembly of *Serratia marcescens* type strain ATCC 13880. *Genome Announc* 2(5) doi:10.1128/genomeA.00967-14
- Dereeper A, Audic S, Claverie JM, Blanc G (2010) BLAST-EXPLORER helps you building datasets for phylogenetic analysis. *BMC evolutionary biology* 10 doi:10.1186/1471-2148-10-8
- Dereeper A, et al. (2008) Phylogeny.fr: robust phylogenetic analysis for the non-specialist. *Nucleic Acids Res* 36:W465-W469 doi:10.1093/nar/gkn180
- Dillman AR, Cronin CJ, Tang J, Gray DA, Sternberg PW (2014) A modified mole cricket lure and description of *Scapteriscus borellii* (Orthoptera: Gryllotalpidae) range expansion and calling song in California. *Environmental Entomology* 43(1):146-156 doi:10.1603/En13152

624 Dillman AR, Guillermin ML, Lee JH, Kim B, Sternberg PW, Hallem EA (2012) Olfaction
 625 shapes host-parasite interactions in parasitic nematodes. *Proc Natl Acad Sci*
 626 USA 109(35):E2324-33 doi:10.1073/pnas.1211436109
 627 Dillman AR, Schneider DS (2015) Defining resistance and tolerance to cancer. *Cell Rep*
 628 13(5):884-7 doi:10.1016/j.celrep.2015.09.052
 629 Edgar RC (2004) MUSCLE: a multiple sequence alignment method with reduced time
 630 and space complexity. *Bmc Bioinformatics* 5:113
 631 Flyg C, Xanthopoulos KG (1983) Insect pathogenic properties of *Serratia marcescens* -
 632 passive and active resistance to insect immunity studied with protease-deficient
 633 and phage-resistant mutants. *J Gen Microbiol* 129(Feb):453-464
 634 Frank JH (2009) *Steinernema scapterisci* as a biological control agent of *Scapteriscus*
 635 mole crickets. In: Hajek AE (ed) Use of microbes for control and eradication of
 636 invasive arthropods. *Progress in Biological Control*, vol 6. Springer, The
 637 Netherlands, p 115-131
 638 Frank JH, Parkman JP (1999) Integrated pest management of pest mole crickets with
 639 emphasis on the southeastern USA. *Integrated pest management reviews* 4:39-
 640 52
 641 García ER (2006) An annotated checklist of some orthopteroid insects of Mapimi
 642 biosphere reserve (Chihuahuan desert), Mexico. *Acta Zoológica Mexicana*
 643 22(3):131-149
 644 Grimont F, Grimont PAD (2006) The genus *Serratia*. *Prokaryotes: A handbook on the*
 645 *biology of bacteria*, Vol 6, Third Edition:219-244 doi:10.1007/0-387-30746-x_11
 646 Guindon S, Gascuel O (2003) A simple, fast, and accurate algorithm to estimate large
 647 phylogenies by maximum likelihood. *Syst Biol* 52(5):696-704
 648 Howick VM, Lazzaro BP (2017) The genetic architecture of defence as resistance to and
 649 tolerance of bacterial infection in *Drosophila melanogaster*. *Mol Ecol* 26(6):1533-
 650 1546 doi:10.1111/mec.14017
 651 Jackson T, Alves S, Pereira R (2000) Success in biological control of soil-dwelling
 652 insects by pathogens and nematodes. In: G G, S W (eds) *Biological control:*
 653 *Measures of success*. Springer, The Netherlands, p 271-296
 654 Kirby JT, Sader HS, Walsh TR, Jones RN (2004) Antimicrobial susceptibility and
 655 epidemiology of a worldwide collection of *Chryseobacterium* spp.: Report from
 656 the SENTRY Antimicrobial Surveillance Program (1997-2001). *J Clin Microbiol*
 657 42(1):445-448 doi:10.1128/Jcm.42.1.445-448.2004
 658 Kurz CL, et al. (2003) Virulence factors of the human opportunistic pathogen *Serratia*
 659 *marcescens* identified by in vivo screening. *Embo J* 22(7):1451-1460 doi:DOI
 660 10.1093/emboj/cdg159
 661 Lemaitre B, Hoffmann J (2007) The host defense of *Drosophila melanogaster*. *Annual*
 662 *review of immunology* 25:697-743
 663 doi:10.1146/annurev.immunol.25.022106.141615
 664 Lin YT, Jeng YY, Lin ML, Yu KW, Wang FD, Liu CY (2010) Clinical and microbiological
 665 characteristics of *Chryseobacterium indologenes* bacteremia. *J Microbiol*
 666 *Immunol* 43(6):498-505 doi:Doi 10.1016/S1684-1182(10)60077-1
 667 Louie A, Song KH, Hotson A, Tate AT, Schneider DS (2016) How many parameters
 668 does it take to describe disease tolerance? *Plos Biology* 14(4)
 669 doi:10.1371/journal.pbio.1002435
 670 Mhina GJ, Leppla NC, Thomas MH, Solis D (2016) Cost effectiveness of biological
 671 control of invasive mole crickets in Florida pastures. *Biological Control* 100:108-
 672 115 doi:10.1016/j.biocontrol.2016.05.017
 673 Nehme NT, et al. (2007) A model of bacterial intestinal infections in *Drosophila*
 674 *melanogaster*. *PLoS Pathog* 3(11):e173 doi:10.1371/journal.ppat.0030173

- Nguyen KB, Smart GC (1990) *Steinernema scapterisci* n. sp. (Rhabditida, Steinernematidae). J Nematol 22(2):187-199
- Nguyen KB, Smart GC (1991) Pathogenicity of *Steinernema scapterisci* to selected invertebrates. J Nematol 23(1):7-11
- Nguyen KB, Smart GC (1992) Life-Cycle of *Steinernema-Scapterisci* Nguyen and Smart, 1990. J Nematol 24(1):160-169
- Nickle DA, Castner JL (1984) Introduced species of mole crickets in the United-States, Puerto-Rico, and the Virgin-Islands (Orthoptera, Gryllotalpidae). Ann Entomol Soc Am 77(4):450-465
- Nickle DA, Frank W (1988) Pest mole crickets, *scapteriscus acletus* (Orthoptera, Gryllotalpidae), reported established in Arizona. Florida Entomologist 71(1):90-91 doi:Doi 10.2307/3494899
- Råberg L, Sim D, Read AF (2007) Disentangling genetic variation for resistance and tolerance to infectious diseases in animals. Science 318(5851):812-4 doi:10.1126/science.1148526
- Rambaut A, Drummond A (2009) FigTree v1.3.1.
- Romano S, et al. (2009) Multilocus sequence typing supports the hypothesis that *Ochrobactrum anthropi* displays a human-associated subpopulation. BMC Microbiology 9 doi:10.1186/1471-2180-9-267
- Samuels RI, Charnley AK, Reynolds SE (1988) The role of destruxins in the pathogenicity of 3 strains of *Metarhizium anisopliae* for the tobacco hornworm *Manduca sexta*. Mycopathologia 104(1):51-58 doi:10.1007/Bf00437924
- Stephan C (1977) Methods for calculating an LC50. In: Mayer F, Hamelink J (eds) Aquatic Toxicology and Hazard Evaluation. American Society for Testing and Material, West Conshohocken, Philadelphia, Pennsylvania, p 65-84
- Torres-Barragan A, Suazo A, Buhler WG, Cardoza YJ (2011) Studies on the entomopathogenicity and bacterial associates of the nematode *Oscieius carolinensis*. Biological Control 59(2):123-129
- Ulagaraj SM (1976) Sound production in mole crickets (Orthoptera-Gryllotalpidae-Scapteriscus). Ann Entomol Soc Am 69(2):299-306
- Ulagaraj SM, Walker TJ (1973) Phonotaxis of crickets in flight: Attraction of male and female crickets to male calling songs. Science 182:1278-1279
- Walker TJ (1984) Biology of pest mole crickets: systematics and life cycles. University of Florida Agriculture Station Bulletin 846:3-10
- Walker TJ, Nickle DA (1981) Introduction and spread of pest mole crickets - *Scapteriscus vicinus* and *Scapteriscus acletus* reexamined. Ann Entomol Soc Am 74(2):158-163
- Xia Y, Hertl PT, Brandenburg RL (2000) Surface and subsurface application of *Beauveria bassiana* for controlling mole crickets (Orthoptera : Gryllotalpidae) in golf courses. J Agr Urban Entomol 17(4):177-189

719 **Tables and Figures**

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

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

721

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

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

<i>Chryseobacterium species</i>	32	30	1	1	1	1	1
<i>Ochrobactrum anthropi</i>	30	32	31	30	29	24	9
<i>Serratia marcescens</i> DB1140	37	30	21	19	15	9.5	1
<i>Serratia marcescens</i> (pond)	35	30	32	29	23	17	1
<i>Beauveria bassiana</i>	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
<i>Serratia marcescens</i> (red)	14.97	-3.841
<i>Serratia marcescens</i> (white)	19.36	-0.905
<i>Serratia marcescens</i> (purple)	3.186	-1.143
<i>Achromobacter xylosoxidans</i>	12.20	-1.417
<i>Chryseobacterium species</i>	12.81	-1.639
<i>Ochrobactrum anthropi</i>	10.9 x 10 ⁷	-0.283
<i>Serratia marcescens</i> DB1140	1043	-0.057
<i>Serratia marcescens</i> (pond)	77,648	-0.541
<i>Beauveria bassiana</i>	1.06	-3.017

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730

733 **Figure 1.** Phylogenetic relationships of bacteria isolated from mole crickets
734 based on maximum likelihood analyses. Bootstrap support values indicated
735 above the branch. Taxon labels are based on best reciprocal hits of the 16S
736 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*.

Figure 3. *In vivo* growth 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*.

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, EC₅₀, 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

757 tolerance. (D) *S. marcescens* (pond) disease tolerance. (E) *Beauveria bassiana*
758 disease tolerance.
759

760 **Figure S1.** Pictures of the microbial cultures isolated from *S. borellii* mole
761 crickets. (A) *S. marcescens* (red). The whitish colonies in this image became red
762 after a few days. (B) *S. marcescens* (white). (C) *S. marcescens* (purple). The
763 colonies took on a purple hue after a few days. (D) *Chryseobacterium* species.
764 (E) *Achromobacter xylosoxidans*. (F) *Ochrobactrum anthropi*. (G) *Tsukamurella*
765 *tryosinosolvens*. (H) *Beauveria bassiana* after 96h. (I) *Beauveria bassiana* after
766 48h.

767

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

772

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