***Bacillus Thuringiensis* Predominated in the Zombie Fungus *Ophiocordyceps Unilateralis* S. L.-Infected Ant Cadavers and Investigation of its Biological Properties**

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Abstract

Animal hosts infected and killed by parasitoid fungi become a nutrient-rich cadaver for saprophytes. Bacteria adapted to colonization of parasitoid fungi can be selected and can predominate in the cadaver, actions that consequently impact the fitness of the parasitoid fungi. In Taiwan, the zombie fungus, *Ophiocordyceps unilateralis sensu lato* (Clavicipitaceae: Hypocreales), was found to parasitize eight ant species, with preference for a principal host, *Polyrhachis moesta*. In this study, ant cadavers grew a fungal stroma that was predominated by *Bacillus thuringiensis*. The bacterial diversity in the principal ant host was found to be lower than the bacterial diversity in alternative hosts, a situation that might enhance the impact of *B. thuringiensis* on the sympatric fungus. The *B. thuringiensis* isolates from fungal stroma displayed higher resistance to a specific naphthoquinone (plumbagin) than *B. thuringiensis* from the environment. Naphthoquinones are known to be produced by *O. unilateralis s. l.*, and hence the resistance displayed by *B. thuringiensis* isolates to these compounds suggests an advantage to *B. thuringiensis* to grow in the ant cadaver. Bacteria proliferating in the ant cadaver inevitably compete for resources with the fungus. However, the *B. thuringiensis* isolates displayed *in vitro* capabilities of hemolysis, production of hydrolytic enzymes, and antagonistic effects to co-cultured nematodes and entomopathogenic fungi. Thus, co-infection with *B. thuringiensis* offers potential benefits to the zombie fungus in killing the host under favorable conditions for reproduction, digesting the host tissue, and protecting the cadaver from being taken over by other consumers. With these potential benefits, *B. thuringiensis* is not entirely a competitor but could also be a synergist in *O. unilateralis s. l.* infections under certain conditions.

Introduction

Fungi and bacteria often live in close proximity and share the same microhabitats. The inevitable competition for limited resources promotes the selection of partners tolerant to the presence of each other. Ecological interactions that are either antagonistic or synergistic can develop. Competition for the same resources enhances the antagonistic relationship in many fungus–bacterium associations¹. However, fungi and bacteria can co-occur synergistically, enhancing the adaptations of each other and consequently forming a co-evolving metaorganism².

The ant-pathogenic fungus, *Ophiocordyceps unilateralis sensu lato*, is a well-known parasitoid that causes manipulated behaviors and subsequent death of the ant hosts³. Spores invade the ant hosts by attaching, germinating, and penetrating the cuticles of foraging ant workers. The fungus lives in a yeast-like state as single cells in the host hemolymph and causes a series of host behavioral manipulations, which is the origin of the name “zombie ant.” These include convulsion, erratic walking, and finally, the host dying after biting onto leaf veins or twigs. The fungus produces hyphae to form a branching network of cells throughout the ant cadaver⁴, exploits resources by lysing the host tissue, and ends the parasitic life cycle with a stroma sprouting from the intersegmental membrane between the head and prothorax of the host, forming the perithecial plates for spreading spores⁵.

During the ~ 1–2 weeks in which hyphal development occurs in the ant cadaver⁵, the fungus is under selective pressure for host resource exploitation and defense against invaders. Animal cadavers in the terrestrial environment are a nutrient-rich resource for saprophytes⁶. The scarcity of cadavers due to patchy distribution, transient nature, and short supply⁷ promotes rapid colonization and consumption of the available resource by bacteria, which results in a quick succession of microbial populations⁸. However, fungal colonization renders the resource unavailable for most bacteria. Fungal products, such as penicillin⁹, usually result in significantly reduced microbial biodiversity. In the case of *O. unilateralis s. l.*-infected ant cadavers, naphthoquinone derivatives are produced by *O. unilateralis* and affect growth of sympatric bacteria negatively^{10–12}. Bacterial communities shaped by fungal colonization have been characterized in various environments and the specific impacts on both organisms have been described². However, bacterial communities in insect cadavers colonized by parasitoid fungi have been described less frequently^{13–14}.

In the present study, we describe the bacterial community in *O. unilateralis s. l.*-infected ant cadavers and examine the biological properties of the predominant bacterial species. In Taiwan, *O. unilateralis s. l.* was found to infect eight sympatric species: seven *Polyrhachis* ant species and *Camponotus punctatissimus*¹⁵. Despite not being as highly host specific as its relatives in Brazil, Japan, North America, and Thailand^{16–19}, Taiwanese *O. unilateralis s. l.* prefer to infect *P. moesta* more than the other seven ant species. With *P. moesta*, Taiwanese *O. unilateralis s. l.* displays better infection performance, including higher prevalence, developmental rate, and efficiency in host behavior manipulation¹⁵. Using a culture-dependent method to capture the bacterial community in the principal host, *P. moesta*, and an alternate sympatric host, *P. wolffi*, we found *B. thuringiensis* to be a predominant species in cadavers of both ant species. These bacterial isolations are hypothesized to be more adapted to fungal colonization due to tolerance to naphthoquinones. We investigated this hypothesis by examining resistance to the fungus' antibiotic effect on bacterial growth. Because the predominant bacterial species occupied more than 35% of the individuals (see results), we further investigated the possible impact on *O. unilateralis s. l.* infection by examining the *B. thuringiensis* isolates for biological properties potentially related to host-killing (hemolysis and presence of pathogenic and antibiotic genes), resource exploitation (capabilities for lysing host tissue), and defense against possible invaders of the host cadaver (lethal effects on scavenger nematodes and antagonism toward entomopathogenic fungi).

Materials And Methods

Sample collection

Samples were collected from an evergreen broadleaf forest in central Taiwan (Lienhuachi Experimental Forest, Nantou County, 23°55'7"N 120°52'58"E) from January 2017 to March 2018; The permission to collect the plants for the study was obtained from the Lianhuachi Research Center, Taiwan Forestry Research Institute, Council of Agriculture, Executive Yuan, Taiwan (Permission no.: 1062272538). The authors confirm that the present study complies with the IUCN Policy Statement on Research Involving

Species at Risk of Extinction and the Convention on the Trade in Endangered Species of Wild Fauna and Flora. Ant cadavers with fungal growth were collected from the canopy of understory plants with a height of < 3 meters. Ant cadavers infected by *O. unilateralis s. l.* were carefully removed by cutting the leaf and placing it into a 50 mL conical centrifuge tube, which was then transported to the laboratory. Only cadavers in which the fungal growth stage preceded the development of perithecia, which theoretically has the highest biological activity, were collected (Fig. 1). In total, 24 infected *P. moesta* and 20 infected *P. wolffi* samples were pooled, respectively, and examined in this study.

Isolation and cultivation of bacteria

Ants on the leaves were first identified to species, and then each ant was carefully placed using tweezers into a sterilized 1.5 mL microcentrifuge tube (see details in Lin et al.¹⁵). Samples were vortexed in 600 µL sterilized water for a few seconds at 3,000 revolutions/min (rpm) using a vortex mixer (AL-VTX3000L, CAE technology Co., Ltd.), and were then soaked with 600 µL 70% ethanol to sterilize the ant's surface. The ethanol on the samples was washed out twice with 600 µL of sterilized water, then finally vortexed in 400 µL of sterilized water. Next, 200 µL of the supernatant was spread homogeneously onto an LB agar plate (25 g Luria-Bertani broth and 15 g agar per liter) to confirm the absence of live bacteria.

Bacteria from the inside of the ant host were released by homogenizing the ant host in 200 µL water and culturing on LB agar plates at 28°C for two days. Approximately 45 (43-46) isolates were randomly picked from each plate with sterile toothpicks, and were suspended in the LB medium supplemented with 15% v/v glycerol and maintained at -80°C until the time of examination. In total, 247 bacterial isolates from *P. moesta* and 241 bacterial isolates from *P. wolffi* were collected.

In addition to the bacterial isolates from the ant bodies, 60 bacterial isolates from soil, leaves, and air in the same forest were collected with the same aforementioned procedure, for the purpose of comparing their resistance to naphthoquinones (see below).

Bacterial identification

Bacteria collected from the ant hosts were identified by gene marker sequencing. The bacterial isolates were cultured in LB medium at 28°C overnight to reach the log-phase, and genomic DNA was extracted following the methods described in Vingataramin and Frost²⁰. Conspecies/strain of the bacterial isolates from the same host was determined using the randomly amplified polymorphic DNA (RAPD) method with the primer 5'-GAGGGTGGCGGTTCT-3'. The PCR amplification was performed as follows: initial denaturation at 95°C for 5 min, 40 cycles of amplification including denaturation at 95°C for 1 min, annealing at 42°C for 30 s, and extension at 72°C for 1 min, followed by a final extension at 72°C for 10 min. PCR products were run in 2% agarose gel and the bacterial isolates were characterized by the fragment patterns. For each of the ant hosts, bacterial isolates with the same RAPD pattern were considered as the same strain. In total, 106 and 178 strains were found from each of the ant hosts, respectively. One of the bacterial isolates was selected at random to represent the strain and coded with JYC followed by a series of numbers. Taxonomic status of each strain was identified to species by the

selected isolates and identifying it using the V3/V4 region of the 16S rDNA gene. PCR amplification with the primer set (8F: 5'-AGAGTTTGATCCTGGCTCAG-3' and 1541R: 5'-AAGGAGGTGATCCAGCCGCA-3')²¹⁻²² was performed under the following conditions: initial denaturation at 95°C for 5 min, 40 cycles of amplification including denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 1 min 45 s, followed by a final extension at 72°C for 10 min. The PCR products were first checked by running a gel, and were then sequenced at Genomics, Inc. (New Taipei City, Taiwan). Taxa for the sequences were judged by the BLAST method against nucleotide sequences in the NCBI database (<https://www.ncbi.nlm.nih.gov/>). Genbank accession numbers of the sequences uploaded to the NCBI database were provided in Supplementary file 1.

The 60 bacterial isolates collected from the environment were examined using the RAPD method and a *Bacillus*-specific primer set (5'-CTTGCTCCTCTGAAGT TAGCGGCG-3' and 5'-TGTTCTTCCCTAATAACAGAGTTTTACGACCCG-3'), with the PCR conditions suggested in Nakano et al.²³. Twenty of the bacterial isolates (10 *Bacillus* and 10 non-*Bacillus*) with different RAPD patterns were collected for further experiments.

Bacterial diversity of the two ant host species

Three biodiversity indexes (Chao1 richness, exponential of Shannon entropy, and inverse Simpson concentration) of bacterial species were estimated by the sample size-based rarefaction/extrapolation sampling curve based on the abundance of bacterial isolates from each of the two ant host species²⁴. The calculation was conducted using R²⁵ with the "iNEXT" package²⁶.

Biological properties of bacterial isolates from infected ants

For examining the biological properties of the most predominant species, *B. thuringiensis* (see results), eleven from 47 and ten from 63 of the *B. thuringiensis* strains from *P. moesta* and *P. wolfei*, respectively, were selected. The strains were selected according to the UPGMA analysis of the sequence. One to three strains grouped in the same cluster were selected (Fig. S1). In addition, six from 15 strains of the second predominant *Bacillus* species (*B. gibsonii*) in *P. wolfei*, as it occupied approximately 20% of the individuals among *Bacillus*, were also randomly selected for the examinations.

All the selected strains were used to examine the biological properties including potential 1) capability of the isolate to lyse host tissue (hydrolytic enzymes); 2) defense against fungal competition for the ant cadaver, presence of pathogenic and antibiotic genes; and 3) resistance to naphthoquinone derivatives. In addition to the repellence against entomopathogenic fungi, one of the *B. thuringiensis* strains from each of the hosts was selected at random for examining the potential impact on the nematode invasion.

Hemolysis reaction

Hemolysis reaction tests were conducted on tryptic soy agar (TSA) plates (15 g pancreatic digest of casein, 5 g soybean meal, 5 g NaCl, and 15 g agar, with final pH of 7.3) mixed with 5% defibrinated sheep

blood, which was added to the TSA after it had cooled down to approximately 50°C. One 3 µL drop of the log-phase bacterial suspension was placed onto each TSA plate and incubated at 28°C for 1-2 days.

The hemolysis reaction was determined by the formation of clean (β-hemolysis) or greenish (α-hemolysis) hemolytic zones, or no such zone (γ-hemolysis, non-hemolytic) around the bacterial colonies²⁷.

Production of hydrolytic enzymes

The production of hydrolytic enzymes was examined by culturing a 3 µL drop of the exponential-phase bacterial suspension on four different types of plated media: chitinase detection medium (solid medium with 0.3 g MgSO₄·7H₂O, 3 g (NH₄)₂SO₄, 2 g KH₂PO₄, 1 g citric acid monohydrate, 0.15 g bromocresol purple, 200 µL Tween 80, 4.5 g colloidal chitin, and 1 L deionized water with 1.5% [w/v] agar and final pH of 4.7); skim milk agar (solid medium with 2% [w/v] agar, 28 g skim milk powder, 5 g casein enzymic hydrolysate (Tryptone), 2.5 g yeast extract, 1 g dextrose, and 1 L deionized water); lipase agar (solid medium with 2% [w/v] agar, 0.1 g phenol red, 1 g CaCl₂, 10 mL olive oil, and 1 L deionized water, with final pH of 7.4); and esterase agar (solid medium with 2% [w/v] agar, 0.1 g phenol red, 1 g CaCl₂, 10 mL tributyrin, and 1 L deionized water, with final pH of 7.4). The chitinase detection medium was used to examine purple zones, indicating chitinase activity²⁸⁻²⁹; the skim milk agar medium was used to examine clearance zones for proteases activity³⁰; and the lipase and esterase agar media were used to examine yellow zones, indicating lipase and esterase activity, respectively³¹.

Pathogenic and antibiotic genes

The total genomic DNA of *Bacillus* strains was extracted by using an AccuPrep genomic DNA extraction kit (Bioneer) for PCR amplification. The specific screening primers for amplifying the genes, including *cry*, *cyt*, Iturin, Chitinase, Bacillomycin, Fengycin, Surfactin, *vip*, and Zwittermicin A, were used under PCR conditions suggested in previous studies³²⁻³⁵. The primer sets used for the amplifications are listed in Table S2-1.

Lethal effects on Caenorhabditis elegans

Antagonistic effects of *B. thuringiensis* isolates on the model nematode, *Caenorhabditis elegans*, were examined by estimating the potential of hemolytic *B. thuringiensis* to prevent competition by scavengers for the resource-rich insect cadavers³⁶. Daily mortality of *C. elegans* strain N2 was examined in response to one random selected *B. thuringiensis* strain (*B. thuringiensis* JYCB227 from *P. moesta* and *B. thuringiensis* JYCB302 from *P. wolfi*) in comparing with one strain of the second predominated *Bacillus* species (*B. drentensis* JYCB252 from *P. moesta* and *B. gibsonii* JYCB395 from *P. wolfi*).

Synchronized L4 nematodes were grown on nematode growth medium (NGM; 3 g NaCl, 2.5 g peptone, 17 g agar, 5 mg cholesterol, 1 mL 1 M CaCl₂, 1 mL 1 M MgSO₄, 25 mL 1 M KH₂PO₄, and H₂O to 1 liter) agar plates seeded with *Escherichia coli* OP50. The *Bacillus* isolates were prepared by inoculating in 3 mL LB

liquid broth at 20°C overnight, and then adjusting to an absorbance of optical density (O.D.) 0.2 at a wavelength of 600 nm.

To test the survival rate of *C. elegans* in the presence of various bacteria, L4 nematodes were co-cultured with 1) a hemolytic bacterial strain, 2) a non-hemolytic bacterial strain, 3) a hemolytic strain + *E. coli* OP50, 4) a non-hemolytic strain + *E. coli* OP50, and 5) *E. coli* OP50 only (control). Twenty µL of bacterial culture was added to a 35 mm NGM agar plate and spread evenly with a glass rod. For each treatment, 30 L4 larvae were cultured on the NGM agar plate and their survival was monitored daily for seven days. Each treatment was replicated three times.

Survival curves were compared using a survival analysis with treatment as the fixed effect. The significance of fixed effect was assessed by model reduction and the likelihood ratio test. Post hoc multiple comparisons were conducted with Tukey's all-pair comparisons. The model building and hypothesis tests were conducted by using the "survival" and "multcomp" packages in R.

Antagonism to entomopathogenic fungi

We examined the response of three entomopathogenic fungi, including *Aspergillus nomius* (isolated from the ant *Dolichoderus thoracicus*), *Trichoderma asperellum* (isolated from the litchi stink bug, *Tessaratomia papillosa*), and *Purpureocillium lilacinum* (isolated from *T. papillosa*), to co-cultured *Bacillus* strains. The entomopathogenic fungi were prepared by culturing on potato dextrose agar (PDA) plates for 4 (*A. nomius*, *T. asperellum*) or 10 (*P. lilacinum*) days at 28°C, until the mycelia covered approximately 80% of the plate.

A piece of mycelium (approximately 5 × 5 mm²) was seeded in the center of a TSA plate and surrounded by three equidistant 3 µL drops of exponential-phase bacterial suspension. Plates were incubated at 20°C for 7-10 days. After incubation, areas of the mycelium occupying the plate surface were photographed and measured using Image J. Each pair of bacteria and entomopathogenic fungi, plus the control (a piece of mycelium not surrounded by the bacterial suspension) was replicated 3-4 times.

Antagonism was estimated based on the percentage of mycelial growth inhibition (MGI), which was calculated using the formula $([R_c - R_{exp}]/R_c) \times 100\%$, where R_c is the mean area of the control fungus and R_{exp} is the mean area of the examined entomopathogenic fungus co-cultured with each of the *Bacillus* strains³⁷. The MGI values among all entomopathogenic fungi were compared using a beta regression model with the *Bacillus* species as the fixed effect. The significance of the *Bacillus* species effect was tested by comparing the full model with a model that removed the fixed effect term by using a likelihood ratio test. Post hoc tests were conducted using a Tukey-adjusted pairwise comparison. The statistical analysis was conducted using the R packages "betareg," "emmeans," "lme4," and "multcomp."

Resistance of bacterial isolates to naphthoquinones

To examine the resistance of bacterial isolates to naphthoquinones, the growth of 11 predominant *B. thuringiensis* strains isolated from the principal ant host was compared with the growth of 20 environmental bacterial isolates (10 *Bacillus* and 10 non-*Bacillus*) using two naphthoquinones, respectively. The two naphthoquinones prepared for the experiment, plumbagin³⁸ and lapachol³⁹, were dissolved in 30% dimethyl sulfoxide (DMSO) water solution³⁸. Naphthoquinone concentrations were determined from the serial dilutions in which three randomly selected bacteria from the ant host and three from the environment had the most distinctive growth rate. Based on these results, concentrations of 45 µg/mL (plumbagin) and 64.5 µg/mL (lapachol) were used (Fig. S2).

In this experiment, the bacterial isolates were first inoculated in LB medium at 20°C overnight and were then refreshed to the exponential phase with LB medium for 3 hours. The bacterial concentration was adjusted to $\sim 1.5 \times 10^8$ cells/mL. Next, 10 µL of the bacterial suspension and 180 µL Mueller Hinton broth medium (Sigma-Aldrich) were added to either 10 µL naphthoquinone solution or 10 µL 30% DMSO water solution for the control. The growth of bacterial isolates at 20°C was monitored by measuring the O.D. value at 600 nm with a Multiskan GO microplate spectrophotometer (Thermo Scientific) every hour for 12 hrs. Each combination of bacterial isolate and naphthoquinone or control was replicated twice. Four bacterial isolates (one *B. thuringiensis* from the ant host, plus two *Bacillus* and one non-*Bacillus* from the environment) were omitted from the analysis due to low growth rate in the media with DMSO (O.D. value lower than 0.05 at the end of 12 hr).

The resistance index of each bacterial isolate was calculated by the normalized difference of the O.D. value in the naphthoquinone-treated medium versus the control medium $([\text{naphthoquinone} - \text{DMSO}]/[\text{naphthoquinone} + \text{DMSO}])$. Values closer to 1 represent higher resistance to the presence of naphthoquinone. Resistance index was compared among the bacterial isolates from different resources (*B. thuringiensis* from the ant host, *Bacillus* from the environment, and non-*Bacillus* from the environment) using a linear mixed model with resource as the fixed effect, bacterial isolate as a random effect, and growth time (5-12 hrs) as a nest effect. The significance of resource as a fixed effect was assessed by model reduction and the likelihood ratio test. Post hoc multiple comparisons were made using Tukey's all-pair comparisons. The model building and hypothesis tests were conducted using the "lme4" and "multcomp" packages in R.

Results

Relative abundance and diversity of cultivated bacteria in infected ant hosts

A total of 247 and 241 bacterial isolates were obtained from infected *P. moesta* and *P. wolfi*, respectively. Firmicutes predominated in both of the infected ant species (95.55% in *P. moesta* and 87.14% in *P. wolfi*), followed by Actinobacteria (4.05% in *P. moesta* and 12.86% in *P. wolfi*) and Proteobacteria (0.40% in *P. moesta* and 0% in *P. wolfi*). At the genus level, Firmicutes were composed of *Bacillus*

(61.54%), *Staphylococcus* (26.32%), *Paenibacillus* (4.86%), *Lysinibacillus* (2.43%), *Terribacillus* (0.40%), and *Streptomyces* (0.40%) in the infected *P. moesta*, and *Bacillus* (71.78%), *Paenibacillus* (4.56%), *Oceanobacillus* (3.73%), *Lysinibacillus* (2.90%), *Sporosarcina* (2.07%), *Staphylococcus* (1.24%), *Rummeliibacillus* (0.41%), and *Virgibacillus* (0.41%) in the infected *P. wolffi*. Actinobacteria were composed of *Gordonia* (3.24%) and *Tsukamurella* (0.40%) in the infected *P. moesta*, and *Kocuria* (7.05%), *Brachybacterium* (3.73%), *Dermacoccus* (0.83%), *Kytococcus* (0.83%), and *Micrococcus* (0.41%) in the infected *P. wolffi*. The sole genus belonging to Proteobacteria was *Brevundimonas* (0.40%) in the infected *P. moesta* (Fig. 2; Table S1). The genus *Bacillus* also contained the most predominant species, *B. thuringiensis*, in both of the hosts (44.94% in *P. moesta* and 36.93% in *P. wolffi*).

The number of bacterial species detected in infected *P. wolffi* (38 species, estimated sample coverage: 94.21%) was more than that in infected *P. moesta* (27 species, estimated sample coverage: 95.97%). Sample size-based rarefaction and extrapolation curves also showed a higher diversity of microbiota in infected *P. wolffi* in all three biodiversity indexes. Figure 2 (S3) suggests an overall increased diversity of host-associated bacterial communities in infected *P. moesta* compared with *P. wolffi*.

Hemolytic activity of bacterial isolates

In the 247 and 241 bacterial isolates from *P. moesta* and *P. wolffi*, respectively, 117 (47.37%) and 110 (45.64%) of them displayed the β -hemolysis reaction. At the species level, 7 of 27 (25.93%) bacterial species from *P. moesta* and 11 of 38 (28.95%) from *P. wolffi* had at least one isolate display the β -hemolysis reaction. Bacterial isolates belonging to four of the nine genera from *P. moesta* displayed hemolytic activity. Among those, 83.33% of the isolates were *Lysinibacillus*, 72.37% were *Bacillus*, 8.33% were *Paenibacillus*, and 1.54% were *Staphylococcus*. Among the bacterial isolates from *P. wolffi*, three of the 13 genera displayed hemolytic activity. Among those, 61.85% of the isolates were *Bacillus*, 18.18% were *Paenibacillus*, and 14.29% were *Lysinibacillus*. Within *Bacillus*, three of 14 species from *P. moesta* and eight of 17 species from *P. wolffi* displayed hemolytic activity (Table S1). None of the isolates showed an α -hemolysis reaction.

Production of hydrolytic enzymes by *Bacillus thuringiensis* and *B. gibsonii*

Among the four hydrolytic enzymes, *B. thuringiensis* isolates from both of the ant hosts displayed protease, lipase, and esterase activities. Chitinase activity was detected in *B. thuringiensis* isolates from *P. wolffi*, but could not be confirmed in the *B. thuringiensis* isolates from *P. moesta* because none of these isolates grew on the chitinase detection medium. Lipase activity was detected in all of the *B. gibsonii* isolates, but none of the isolates grew on either the chitinase detection medium, skim milk agar (for protease activity), or esterase agar plates (Table S3).

PCR-based screening of endotoxin-related and biosynthetic genes

Most of the pathogenic and antibiotic genes examined in the current study were not commonly detected in the 11 *B. thuringiensis* strains from *P. moesta*, 10 *B. thuringiensis* strains from *P. wolffi*, or six *B. gibsonii*

strains from *P. wolffi*. In the examination of *cry* genes, one *B. gibsonii* strain displayed the signal for the *cry2* gene, and two *B. thuringiensis* strains from *P. moesta* displayed the signal for the *cry3* gene. The chitinase gene signal was displayed in four *B. thuringiensis* strains from *P. moesta*, two *B. thuringiensis* strains from *P. wolffi*, and one *B. gibsonii* strain. The Surfactin gene signal was displayed in four *B. thuringiensis* strains from *P. moesta*. The Zwittermicin A gene signal was displayed in one *B. thuringiensis* strain from *P. moesta*. The other five genes examined (*cyt*, Iturin, Bacillomycin, Fengycin, and *vip*) were not detected in any of the *Bacillus* strains (Table S2-2, S2-3).

Potential impact of *B. thuringiensis* on *Caenorhabditis elegans* fitness

Hemolytic *B. thuringiensis* from both of the ant hosts displayed higher mortality to nematodes than did the coexisting non-hemolytic *Bacillus*, whereas nematode survival in the presence of non-hemolytic *Bacillus* was similar to that of the *E. coli* OP50 control (*P. moesta*: $X^2 = 80.14$, *d.f.* = 4, $P < 0.001$, Fig. 3a; *P. wolffi*: $X^2 = 46.39$, *d.f.* = 4, $P < 0.001$, Fig. 3b). The addition of *E. coli* OP50 slightly increased the survival rate of nematodes co-cultured with hemolytic *B. thuringiensis* isolated from *P. wolffi* (Fig. 3b), but this impact was not seen when using hemolytic isolates from *P. moesta* (Fig. 3a).

Growth inhibition of entomopathogenic fungi by *Bacillus* isolates

All three entomopathogenic fungi displayed growth inhibition when they were co-cultured with *B. thuringiensis* from either host species, or with *B. gibsonii* (*A. nomius*: $X^2 = 87.21$, *d.f.* = 2, $P < 0.001$; *T. asperellum*: $X^2 = 51.06$, *d.f.* = 2, $P < 0.001$; *P. lilacinum*: $X^2 = 76.33$, *d.f.* = 2, $P < 0.001$). The *B. thuringiensis* from each of the ant hosts displayed similar growth inhibition of the entomopathogenic fungi, whereas *B. gibsonii* had significantly lower growth inhibition (Fig. 4).

Resistance of *Bacillus thuringiensis* to naphthoquinones

The bacteria we examined responded differently to the two naphthoquinones used in this study. Bacterial isolates of all three categories (*B. thuringiensis* from the ant hosts, *Bacillus* from the environment, and non-*Bacillus* from the environment) displayed similar resistance to lapachol ($X^2 = 1.87$, *d.f.* = 2, $P = 0.392$, Fig. 5a), growing similarly to bacteria cultured in the control medium (resistance indexes close to 1). In contrast, bacteria isolated from the environment, particularly the *Bacillus*, grew much slower in the presence of plumbagin than in the control medium, whereas *B. thuringiensis* from the ant host displayed a higher resistance to plumbagin ($X^2 = 6.91$, *d.f.* = 2, $P = 0.0316$, Fig. 5b).

Discussion

We found that *B. thuringiensis*, which occupied more than 35% of the total bacterial counts, predominated the bacterial community in *O. unilateralis* s. l.-infected ant cadavers. In a study of the closely related fungus, *O. sinensis*, the *Ophiocordyceps*-associated microbiomes improved the development and formation of fungal metabolites^{13–14}. However, the main bacterial taxa found in the *O. sinensis*-infected host cadavers was not *Bacillus*^{13–14}. In the present study, the proliferation of *Bacillus* in

ant cadavers could be from an invasion of the naphthoquinone-resistant population from soil. The genus *Bacillus* is one of the main bacterial groups in soil⁴⁰. Furthermore, the predominant *B. thuringiensis* from ant cadavers displayed higher resistance to a specific naphthoquinone (plumbagin) than the bacteria isolated from the surrounding environment. Although such differences in the naphthoquinone effect have not been seen in lapachol, unequal antimicrobial activity has been found in previous studies (e.g.⁴¹). In addition, the *B. thuringiensis* isolates from ant cadavers displayed hydrolytic enzyme activity, which suggests the potential to exploit resources of ant cadavers. Not surprisingly, lower bacterial diversity resulted in a higher abundance of *B. thuringiensis* in cadavers of the principal host, *P. moesta*, in comparison with the alternate sympatric host, *P. wolffi*. The accumulation of naphthoquinones might open a niche for *B. thuringiensis* isolates to out compete other saprophytes, but the lower bacterial diversity in the principal host suggests a more sophisticated manipulation of the physiological conditions of the principal host. Despite not diverging among its sympatric hosts, *O. unilateralis s. l.* displays higher infection performances in one ant species than in the other host species in Taiwan¹⁵. The efficient exploitation of host resources may result from better modification of host physiological conditions, which consequently causes a more uniform environment for specific bacterial species. Fungi are known to modify the environment for their own benefit². Some of the biological properties of the *B. thuringiensis* isolates, including *in vitro* hemolysis and antagonistic effects on co-cultured entomopathogenic fungi and nematodes, suggest the potential of a synergistic relationship with *O. unilateralis s. l.* However, because artificial infection of the ant host with *O. unilateralis s. l.* is difficult under our current laboratory conditions, to date we can only hypothesize about the potential of synergistic effects of *B. thuringiensis* on *O. unilateralis s. l.* infections.

The *B. thuringiensis* isolates from both principal and alternate sympatric hosts are broadly aggressive to potential saprophytic invaders, including fungi and nematodes. One possible factor is their hemolytic ability^{42–43}. In comparison with non-hemolytic *Bacillus*, hemolytic *B. thuringiensis* isolates displayed higher fatal effects to free-living nematodes and growth inhibition of co-cultured entomopathogenic fungi. Collaborative bacteria have been known to assist parasites in occupying the host body by excluding potential invaders, such as nematode scavengers or insect predators^{36, 44–45}. For these collaborative bacteria, some of the antibiotic activity against invaders may be moderated by the need to compete with invaders for the necessary nutrition⁴⁶, while not obviously affecting the host's survival⁴⁷. In contrast, for the *B. thuringiensis* isolates, in which the host survival is no more a concern, the antibiotic activity could be more intensive and efficient. The *B. thuringiensis* isolates might play a role resembling that of the symbiotic bacteria, *Xenorhabdus* and *Photorhabdus*, in entomopathogenic nematodes. Invasion of the insect host by the nematode rapidly causes the insect's death, and septicemia occurs with the proliferation of symbiotic bacteria. The nematodes then colonize the insect cadaver with the symbiotic bacteria, which now serve as "body guards" to defend against invasion by saprophytic or parasitic organisms⁴⁵. Like the *B. thuringiensis* isolates, hemolysis is also detected in *X. nematophila* and likely plays a critical role in killing and preserving the insect host⁴⁸. The outbreak of hemolytic *B. thuringiensis* isolates can be lethal to the ant host, and might explain the lack of endotoxin-related and

biosynthetic genes due to overlapping functions. Another explanation for the lack of endotoxin-related genes is that the proliferation of *B. thuringiensis* occurs after the host death. However, the precise timing of the outbreak is currently unknown.

Regardless of the timing of *B. thuringiensis* proliferation, *B. thuringiensis* is potentially beneficial to *O. unilateralis* s. l. in protecting and consuming the host cadaver. *Bacillus* species produce several extracellular enzymes^{49–50}, including alkaline proteases, which have been used commercially⁵¹. The ant cadaver contains nutritious and protein-rich niches for microbiota, but these are only made available in the presence of proteolytic enzymes, which digest the macromolecular proteins into smaller peptides and free amino acids⁵². The proteases activity detected in all of the examined *B. thuringiensis* isolates suggests that symbiotic bacteria are advantageous to *O. unilateralis* s. l. in digesting the host tissue. In addition to improving consumption of the host, proteases are important for host epidermal decomposition and enhanced virulence to assist *O. unilateralis* s. l. in causing host death^{53–54}. Chitin is another major component of insects that functions as a scaffolding material⁵⁵. In addition to releasing nutrients, digesting chitin can be also fatal to the insect host and its invaders. Chitin is a necessary component of the peritrophic matrix secreted by the entire midgut in most insects, and it functions as a protective barrier against abrasive particles and microbial infections⁵⁶. Chitinase secreted by bacteria weakens the insect's peritrophic membrane, and consequently promotes the penetration of bacterial toxins to the gut epithelia during pathogenesis^{57–58}. Chitinase produced by bacteria has also been found to present antagonistic activity against fungi, given that chitin is a primary component of fungal cell walls⁵⁹. Digestion of lipids, in contrast to proteases and chitinase, might be less harmful for the living ant because lipids function mainly as storage structures. Nevertheless, lipids are a concentrated source of energy and primary nutrient reserves for fungal spores⁶⁰. Extracellular lipid digestion by *B. thuringiensis* suggests that this bacterium efficiently harvests the energy from the ant cadaver and can symbiotically benefit *O. unilateralis* s. l. The hydrolytic enzymes, secreted to the microenvironment, could serve as public goods and enhance the utilization of host resources. The importance of *B. thuringiensis* in consuming the host might be further supported by phylogenetic analysis of enzyme sequences. Unlike the genes for producing secondary metabolites, genes for producing hydrolytic enzymes in *O. unilateralis* s. l. display lower species specificity, which suggests a lack of positive selection among host species⁶¹. Sharing of the task by sympatric microorganisms reduces the indispensability and selective pressure on fungal hydrolytic enzymes.

The biological properties associated with an outbreak of *B. thuringiensis* suggest potential benefits to *O. unilateralis* s. l. However, the proliferation of *B. thuringiensis* appears coincidental rather than the result of long-term coevolution. In addition, the net effect of the inevitable competition with *B. thuringiensis* for limited resources may prove to be antagonistic rather than synergistic. Co-occurrence of fungi and bacteria can promote the growth of both entities⁶², but it can also accelerate the depletion of resources. Tradeoffs have been reported in fungi between growth and tolerance toward bacteria¹. In this study, we demonstrated the predominance of *B. thuringiensis* in the bacterial community associated with ant cadavers infected by *O. unilateralis* s. l. These bacterial isolates displayed the capabilities of hemolysis,

production of hydrolytic enzymes, antagonistic effects to co-cultured nematodes and entomopathogenic fungi, and higher tolerance toward naphthoquinone. At present we still do not have an evidence to conclude the outbreak of *B. thuringiensis* is antagonistic or synergistic toward *O. unilateralis* s. l. However, study of sympatric bacteria will improve our understanding of the parasitic life history and potential selective pressures in *O. unilateralis* s. l. In addition, the antibiotic activity of *B. thuringiensis* isolates has potential as a biocontrol agent. With antagonistic effects on entomopathogenic fungi and nematodes, *B. thuringiensis* also has potential agricultural applications in controlling pathogenic fungi⁶³ and root-knot nematodes⁶⁴. Through behavioral manipulation of the *O. unilateralis* s. l.–ant parasitic association, the bacterial diversity revealed in this study is a step forward in understanding the impact of microbial communities in parasitic life cycles.

Declarations

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Figures

Figure 1

Ophiocordyceps unilateralis sensu lato-infected (a) *Polyrhachis moesta* and (b) *P. wolfi*, with the stroma growing from the ant cadaver.

Figure 2

Species diversity and genus abundance of bacteria isolated from ant cadavers infected by *Ophiocordyceps unilateralis sensu lato*.

Figure 3

Daily cumulative mortality rate ($\% \pm \text{SE}$) of the nematode, *Caenorhabditis elegans*, co-cultured with hemolytic and non-hemolytic bacteria isolated from ant cadavers of (a) the principal host, *Polyrhachis moesta*, and (b) the alternative host, *P. wolffi*, of *Ophiocordyceps unilateralis sensu lato*.

Figure 4

Mycelial growth inhibition (MGI) value of three entomopathogenic fungi under the effects of three predominant *Bacillus* strains isolated from ant cadavers infected by *Ophiocordyceps unilateralis sensu lato*. Letters indicate significant pairwise differences among the *Bacillus* species (Tukey's all-pair comparisons using the beta regression model, $p < 0.05$).

Figure 5

The resistance index of bacterial growth in the presence of two naphthoquinones, lapachol (a) and plumbagin (b). Letters indicate significant pairwise differences between *Bacillus thuringiensis* isolated from ant cadavers of *Polyrhachis moesta*, *Bacillus* isolates from the environment, and non-*Bacillus* isolates from the environment (Tukey's all-pair comparisons using the linear mixed model, $p < 0.05$).

Supplementary Files

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- [SupplementaryFigure1.tif](#)

- [SupplementaryFigure2.tif](#)
- [SupplementaryFigure3.tif](#)
- [Supplementaryfile1.csv](#)
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