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Burkholderia from Fungus Gardens of Fungus-Growing Ants Produces Antifungals That Inhibit the Specialized Parasite *Escovopsis*

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ABSTRACT Within animal-associated microbiomes, the functional roles of specific microbial taxa are often uncharacterized. Here, we use the fungus-growing ant system, a model for microbial symbiosis, to determine the potential defensive roles of key bacterial taxa present in the ants' fungus gardens. Fungus gardens serve as an external digestive system for the ants, with mutualistic fungi in the genus *Leucoagaricus* converting the plant substrate into energy for the ants. The fungus garden is host to specialized parasitic fungi in the genus *Escovopsis*. Here, we examine the potential role of *Burkholderia* spp. that occur within ant fungus gardens in inhibiting *Escovopsis*. We isolated members of the bacterial genera *Burkholderia* and *Paraburkholderia* from 50% of the 52 colonies sampled, indicating that members of the family *Burkholderiaceae* are common inhabitants in the fungus gardens of a diverse range of fungus-growing ant genera. Using antimicrobial inhibition bioassays, we found that 28 out of 32 isolates inhibited at least one *Escovopsis* strain with a zone of inhibition greater than 1 cm. Genomic assessment of fungus garden-associated *Burkholderiaceae* indicated that isolates with strong inhibition all belonged to the genus *Burkholderia* and contained biosynthetic gene clusters that encoded the production of two antifungals: burkholdine1213 and pyrrolnitrin. Organic extracts of cultured isolates confirmed that these compounds are responsible for antifungal activities that inhibit *Escovopsis* but, at equivalent concentrations, not *Leucoagaricus* spp. Overall, these new findings, combined with previous evidence, suggest that members of the fungus garden microbiome play an important role in maintaining the health and function of fungus-growing ant colonies.

IMPORTANCE Many organisms partner with microbes to defend themselves against parasites and pathogens. Fungus-growing ants must protect *Leucoagaricus* spp., the fungal mutualist that provides sustenance for the ants, from a specialized fungal parasite, *Escovopsis*. The ants take multiple approaches, including weeding their fungus gardens to remove *Escovopsis* spores, as well as harboring *Pseudonocardia* spp., bacteria that produce antifungals that inhibit *Escovopsis*. In addition, a genus of bacteria commonly found in fungus gardens, *Burkholderia*, is known to produce secondary metabolites that inhibit *Escovopsis* spp. In this study, we isolated *Burkholderia* spp. from fungus-growing ants, assessed the isolates' ability to inhibit *Escovopsis* spp., and identified two compounds responsible for inhibition. Our findings suggest that

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Burkholderia spp. are often found in fungus gardens, adding another possible mechanism within the fungus-growing ant system to suppress the growth of the specialized parasite *Escovopsis*.

KEYWORDS antifungal, attine, burkholderia, burkholdine, defensive symbiosis, escovopsis, fungus-growing ant, pyrrolnitrin

Symbiotic associations are ubiquitous. Organisms do not live in isolation; rather, they exist in complex communities consisting of variable macro- and microorganisms. These symbiotic interactions are known to play a fundamental role in shaping life on earth, and associations can range from transient to obligate and parasitic to beneficial (1). Microbial symbioses span this range and fill a variety of important roles within hosts. Substantial work has been done specifically in association with insect hosts. Research on beneficial microbes in insects has typically focused on the role of symbionts in providing nutrients to their host (2, 3). However, in recent years, it has become clear that microbes often play a critical role in mediating interactions between insects and pathogens or parasites (4, 5). Microbes can provide defense through competitively excluding pathogenic microbes, priming the host immune system, and producing compounds that protect the host (6–8). In this study, we used the fungus-growing ant system to explore a bacterially mediated antifungal defensive symbiosis.

Fungus-growing ants are a well-studied example of a multipartite symbiosis (Fig. 1). Fungus-growing ants (Hymenoptera, Formicidae, Attini, Attina) thrive in the Neotropics and consist of 20 genera and approximately 250 species (9, 10) that have formed ancient and highly evolved symbioses with both fungi and bacteria (11). The ants cultivate fungi in the genus *Leucoagaricus* (Basidiomycota, Agaricales, Agaricaceae) as a food source. Fungus-growing ants bring an organic substrate to structures known as fungus gardens (Fig. 1B), where *Leucoagaricus* spp. principally degrade the substrate and produce usable energy for the ants (12, 13). In addition, a consistent bacterial community composed primarily of *Proteobacteria* exists within fungus gardens (14–18). In leaf-cutter ants, the bacterial community has been shown to help degrade plant secondary compounds and aid in nitrogen acquisition for the ant through biological nitrogen fixation (19, 20). Though the functions of some of the bacterial taxa have been described, the roles of many bacterial fungus garden members are unknown.

Fungus gardens are threatened by a specialized pathogen, the parasitic fungus *Escovopsis* (Ascomycota, Hypocreales, Hypocreaceae) (21). In many lineages of fungus-growing ants, the growth of *Escovopsis* spp. is inhibited by *Actinobacteria* in the genus *Pseudonocardia*, defensive symbionts found on the exoskeleton or occurring within specialized structures on the ants (22–24). However, in the two fungus-growing ant genera *Atta* and *Sericomyrmex*, this defensive symbiosis with *Pseudonocardia* has been secondarily lost (23, 24). Despite the lack of the symbiont *Pseudonocardia*, the fungus gardens are not overrun with *Escovopsis* spp., suggesting other ways of controlling *Escovopsis* spp. The ant behaviors of weeding and grooming are known to be crucial for suppressing *Escovopsis* spp. (25, 26). In addition, there is evidence that antifungal-producing bacteria may colonize the fungus garden and provide some level of inhibition against *Escovopsis* spp. (27). Santos and colleagues (27) isolated *Burkholderia* spp. consistently from *Atta sexdens* fungus gardens (32/57 colonies) and identified isolates that could inhibit the growth of *Escovopsis weberi*, suggesting a potential role for garden bacteria in the defense of fungus gardens.

The fungus gardens' *Proteobacteria*-dominant bacterial community is known to include members of the family *Burkholderiaceae* (14–18, 27). *Burkholderiaceae* have diverse metabolic capabilities and inhabit a broad range of ecological niches (28). Members of two closely related genera within the *Burkholderiaceae*, *Burkholderia* and *Paraburkholderia*, have been found and characterized in the context of mammalian and plant pathogenesis, nitrogen fixation, bioremediation, and plant growth stimulation and/or in close association with fungi

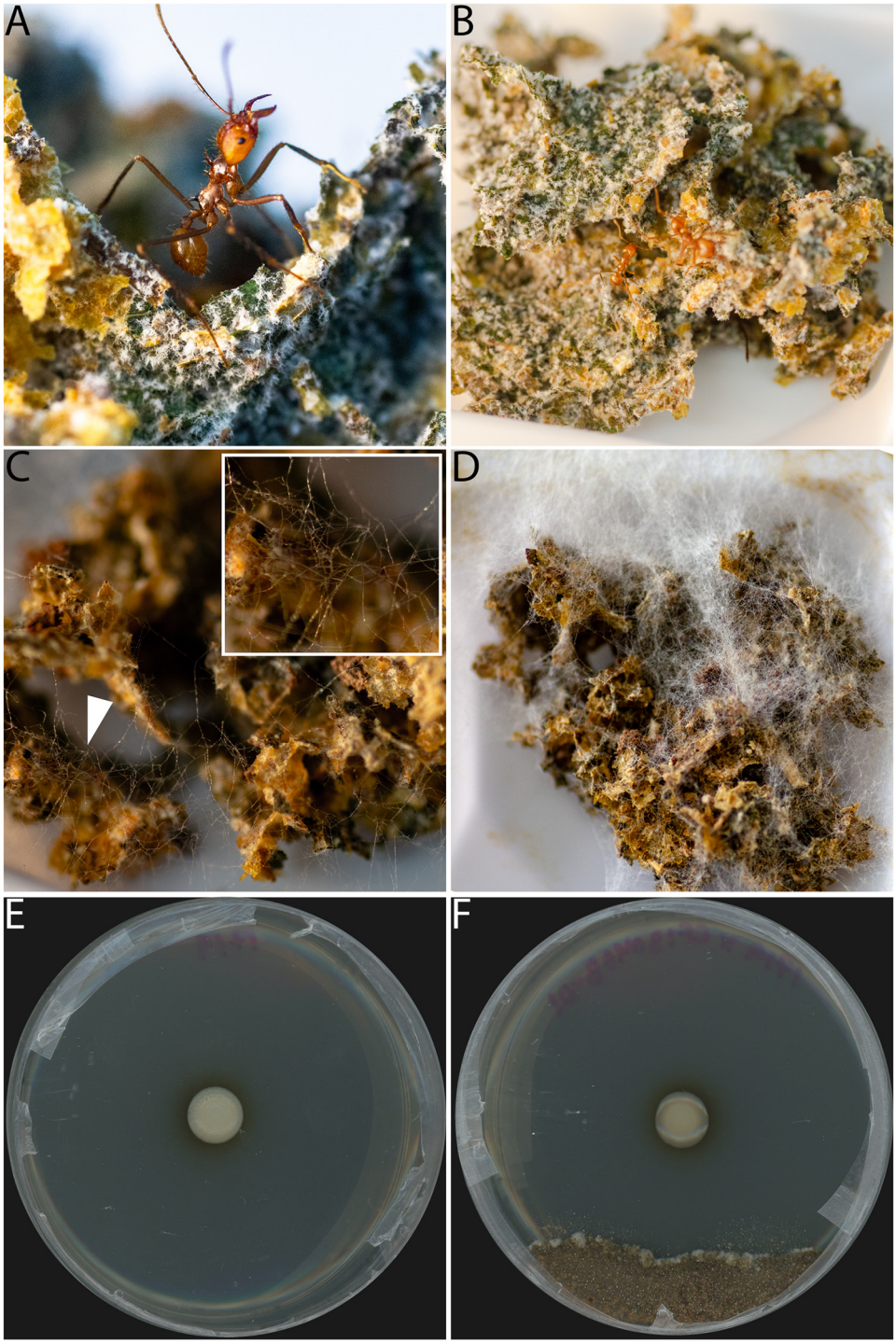

FIG 1 Infection of an *Atta cephalotes* colony by *Escovopsis weberi* (CF180408-01) and *in vitro* petri plate antimicrobial inhibition bioassay with ICBG1719. (A, B) Healthy fungus garden with no *Escovopsis* infection; (C) day 3 postinfection of fungus garden with *Escovopsis*; (D) day 7 postinfection of fungus garden with *Escovopsis*; (E and F) petri plate with only *Burkholderia* sp. ICBG1719 growing (E) and petri plate inhibition bioassay of ICBG1719 against *Escovopsis weberi* (CF180408-01), with a clear zone of inhibition (F). Pictures in panels A to D were taken by Caitlin Carlson.

and insects. These symbiotic *Burkholderia* and *Paraburkholderia* spp. can also produce secondary metabolites that are important in ecological interactions. For example, *Paraburkholderia rhizoxinica* resides in the hyphae of the fungal plant pathogen *Rhizopus microsporus*. *P. rhizoxinica* produces an antimitotic macrolide that is converted into rhizoxin, which is the causative agent

TABLE 1 Summary of ant colony collections by geographic location

Ant genus	No. of colony collections in[a]:	
	Brazil	Costa Rica
Atta[b]	10 (2)	11 (7)
Acromyrmex	3 (0)	1 (0)
Paratrachymyrmex	5 (3)	2 (2)
Mycetomoellerius	0	2 (1)
Sericomyrmex[b]	0	5 (5)
Mycetophylax	2 (2)	0
Cyphomyrmex	1 (0)	0
Myrmicocrypta	1 (0)	0
Apterostigma	5 (3)	0
Unidentified Attini	4 (1)	0
Total colonies	31 (11)	21 (15)

[a]The number of colonies that had a *Burkholderiaceae* isolate is indicated in parentheses.
[b]Two genera, *Atta* and *Sericomyrmex*, have secondarily lost *Pseudonocardia*.

of rice seedling blight (29, 30). Lagriinae beetles depend on *Burkholderia* symbionts for the protection of their eggs. Specifically, *Burkholderia gladioli* produces a blend of antibiotics, including toxoflavin, caryoynencin, lagriene, and sinapigladioside, which protect the egg stage of the beetles against pathogenic microbes (31, 32). Additionally, *Burkholderia* isolates that produce pyrrolnitrin, a characterized antifungal, have been used as biocontrol agents for plant fungal pathogens (33). Finally, as mentioned previously, the study by Santos and colleagues (27) suggests that antifungal-producing *Burkholderia* spp. may play a role in suppressing the fungus garden parasite *Escovopsis weberi* in *Atta sexdens*.

Here, we conduct a comprehensive investigation of the functional role of fungus garden-associated *Burkholderiaceae* isolates obtained from colonies of fungus-growing ants that span major clades in the basal and derived lineages to test the hypothesis that fungus garden-associated bacteria can provide protection against the system's specialized parasites, *Escovopsis* spp. First, we sampled 52 fungus-growing ant fungus gardens that span eight different ant genera to isolate *Burkholderiaceae*. We then tested the ability of a subset of *Burkholderiaceae* isolates to inhibit a panel of 11 fungi, including eight strains of the specialized parasite *Escovopsis* that were isolated from five different genera of fungus-growing ants. To identify potential biosynthetic gene clusters (BGCs) involved in *Escovopsis* inhibition, we sequenced the genomes of 30 *Burkholderiaceae* isolates and performed antiSMASH and BiG-SCAPE analyses. Then, to confirm production of antifungals, organic extracts were prepared from a subset of isolates and analytical chemistry techniques were used to identify antifungals. Finally, the organic extracts were also used to assess the inhibition of *Escovopsis* spp. and six strains of *Leucoagaricus* spp.

RESULTS

***Burkholderiaceae* are consistently present in the fungal gardens of different lineages of attine ants.** *Burkholderiaceae* were frequently isolated from fungus gardens of fungus-growing ants, indicating that they are common residents of fungus gardens. Nutrient-rich, nonselective medium was used for the Brazilian fungus garden bacterial isolations, and 35% of colonies contained at least one *Burkholderiaceae* isolate (Table 1). *Burkholderiaceae* selective medium was used for Costa Rican fungus garden bacterial isolations, and 71% of colonies contained at least one *Burkholderiaceae* isolate (Table 1).

In total, 86 isolates were obtained from these 52 ant colonies, and whole 16S rRNA gene sequences aligned with *Burkholderia* spp. as the top BLAST hit (see Data Set S1 in the supplemental material) for all isolates. We selected a subset of 30 isolates for whole-genome sequencing that represented unique species-level 16S rRNA gene BLAST hits, such that if two isolates from the same fungus garden sample matched the same species, one was randomly selected for sequencing (Data Set S2). Phylogenetic and average nucleotide identity

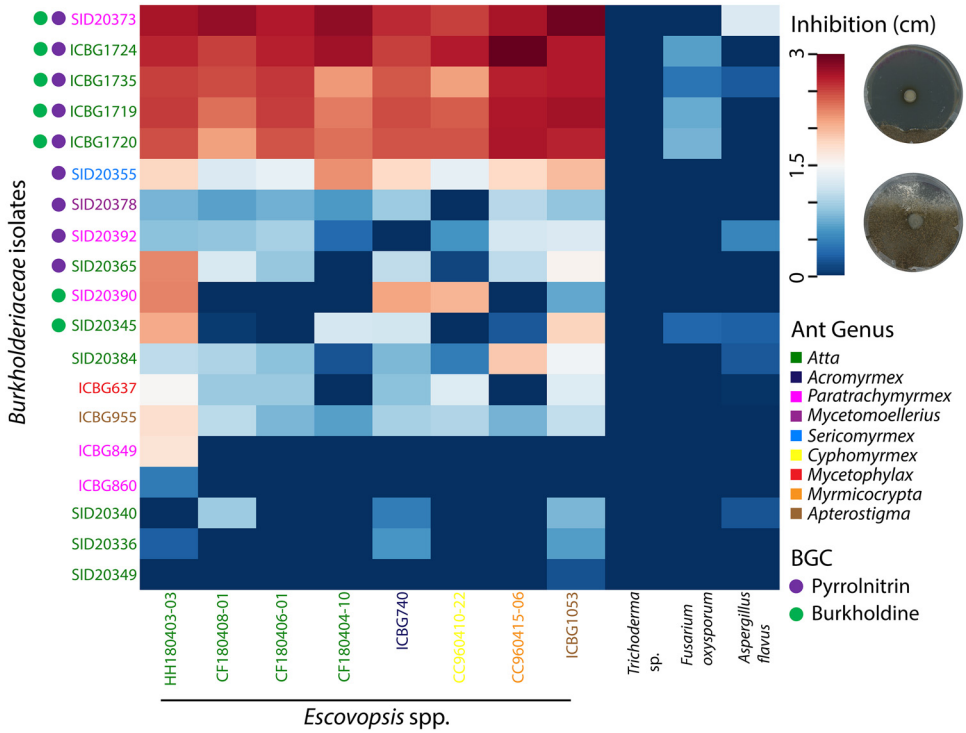

FIG 2 *Burkholderiaceae-Escovopsis* petri plate antimicrobial inhibition bioassays indicated varied levels of inhibition of *Escovopsis* spp. Zones of inhibition (ZOIs) were measured 13 days after inoculation with *Escovopsis* spp. *Burkholderiaceae* and *Escovopsis* isolates are color coded by the ant colony from which they were isolated. Circles to the left of the *Burkholderiaceae* isolates indicate the presence of pyrrolnitrin (purple) or burkholdine (green) biosynthetic gene clusters.

(ANI) analyses indicated that isolates grouped with both *Burkholderia* species and *Paraburkholderia* species (Fig. S1; Data Set S3), in contrast with the original BLAST search using the 16S rRNA gene. Both 16S rRNA gene sequences and whole-genome phylogenies (Fig. S1) indicate that the bacterial isolates from fungus gardens fall among multiple clades, including different lineages within *Burkholderia* spp., such as plant pathogens (*B. gladioli*), the *B. cepacia* complex, and nitrogen-fixing and plant-associated (*B. mimosarum*, *B. nodosa*, *B. xenovorans*, *B. phytofirmans*), as well as plant-, rhizosphere-, and soil-associated, *Paraburkholderia* species. ANI analysis confirmed the variety of *Burkholderiaceae* in fungus gardens. Eighteen out of 30 sequenced isolates shared ≥95% ANI with *B. gladioli* (5/18), *Burkholderia lata* (9/18), *Burkholderia ambifaria* (1/18), *B. cepacia* (1/18), *Burkholderia seminalis* (1/18), or *Paraburkholderia tropica* (1/18), indicating a range of different characterized *Burkholderiaceae* species. The other 12 isolates shared between 89% and 94% ANI with other *Burkholderiaceae* isolates, such as *Burkholderia ubonensis*, *Burkholderia pyrrocinia*, *Paraburkholderia eburnea*, and *Paraburkholderia caribensis* (Data Set S3).

Multiple fungus garden *Burkholderiaceae* isolates inhibit *Escovopsis* spp. In order to assess the potential of fungus garden-associated *Burkholderiaceae* to inhibit *Escovopsis* spp., an *in vitro* petri plate antimicrobial inhibition bioassay with 19 genome-sequenced *Burkholderiaceae* isolates from Brazil and Costa Rica was conducted against a panel of 11 fungi: 8 *Escovopsis* isolates, *Aspergillus flavus* (Ascomycota, Eurotiales, Trichocomaceae), *Fusarium oxysporum* (Ascomycota, Hypocreales, Nectriaceae), and *Trichoderma* sp. (Ascomycota, Hypocreales, Hypocreaceae). After 13 days of coincubation, all 19 *Burkholderiaceae* isolates had at least one zone of inhibition (ZOI) greater than 0 cm against at least one *Escovopsis* strain (Fig. 2; Fig. S2 and S3). Another set of *in vitro* antimicrobial inhibition bioassays with 13 additional isolates from Costa Rican *Sericomyrmex* colonies were conducted against a panel of six *Escovopsis* strains and the same three

non-*Escovopsis* fungi noted above. Similar results were found with the 13 additional isolates; all 13 isolates had at least one ZOI greater than 0 cm against at least one *Escovopsis* strain (Fig. S3). *Burkholderia* isolates SID20373, ICBG1719, ICBG1720, ICBG1724, and ICBG1735 inhibited all *Escovopsis* strains significantly more ($P < 0.001$; average zone of inhibition, $\geq$ 2.45 cm) (Fig. 2) than the other 27 *Burkholderiaceae* isolates surveyed (Fig. 2; Fig. S3; Table S1). For clarity, the five isolates listed above that inhibited *Escovopsis* spp. strongly will be referred to as strong inhibitory isolates. *Aspergillus flavus*, *Trichoderma* sp., and *F. oxysporum*, were not inhibited by any of the *Burkholderiaceae* isolates.

Strong inhibitory *Burkholderia* isolates are predicted to have two antifungal BGCs. To identify secondary metabolites that might be responsible for the inhibitory activity, the sequenced genome of each *Burkholderiaceae* isolate was submitted to antiSMASH v4.0 for the detection of biosynthetic gene clusters (BGCs), groupings of genes that encode the production of a secondary metabolite. BiG-SCAPE was used to compare the presence and absence of BGCs across isolates and to search for correlations between the presence of BGCs and *Escovopsis* inhibition (Fig. S4). Two BGCs were identified in the genomes of all five of the strong inhibitory *Burkholderia* isolates. These two BGCs had high similarity by BLASTP (>85% identity) to BGCs in the MIBiG database: the BGC for pyrrolnitrin and the BGC for occidiofungin (Fig. S4). Pyrrolnitrin is an antifungal alkaloid biosynthesized from tryptophan and initially isolated from *Pseudomonas* spp. (34). Occidiofungin is a hybrid nonribosomal peptide/polyketide antifungal glycopeptide and an analog of the burkholdines (35, 36) (Fig. 3). *Burkholderia* species isolates that did not strongly inhibit *Escovopsis* contained BGCs for only pyrrolnitrin (SID20355, SID20378, SID20392, SID20365), only antifungal glycopeptides (SID20390, SID20345), or neither of these BGCs. None of the *Paraburkholderia* species isolates (SID20336 and others) demonstrated inhibition against *Escovopsis* spp. and were not predicted to contain either antifungal BGC. The distribution of the two antifungal-encoding biosynthetic gene clusters across *Burkholderia* isolates is demonstrated in Fig. 2.

***Burkholderia* extracts containing pyrrolnitrin and burkholdine1213 replicate the results of the petri plate inhibition assay.** To determine if the antifungal compounds were being produced by the strong inhibitory isolates, we made organic extracts from *Burkholderia* isolates predicted to have both of the BGCs that encode the production of these compounds (ICBG1719, ICBG1720, ICBG1724, ICBG1735, SID20373) or one or the other compound (SID20365, pyrrolnitrin; SID20345, antifungal glycopeptides). As a negative control, we also included the *Paraburkholderia* isolate ICBG849, as its genome does not contain either of the BGCs. The extracts were analyzed by ultra-high-performance liquid chromatography high-resolution electrospray ionization mass spectrometry (UPLC-HRESIMS) for the detection of pyrrolnitrin and antifungal glycopeptides. Pyrrolnitrin was identified by its characteristic two-chlorine isotope pattern and by comparison with a pyrrolnitrin standard purchased from Millipore-Sigma. Burkholdine1213, an antifungal glycopeptide closely related to occidiofungin, was identified by comparison to the published molecular weight and molecular formula of $C_{52}H_{83}N_{11}O_{22}$ (36). Pyrrolnitrin was identified in ICBG1719, ICBG1720, ICBG1724, ICGB1735, SID20373, and SID20365. Burkholdine1213 was identified in ICBG1719, ICBG1720, ICBG1724, ICBG1735, SID20373, and SID20345.

After the compounds were identified in the extracts, we tested the extracts against one *Escovopsis weberi* isolate (CF180408-01), *A. flavus*, *F. oxysporum*, and *Trichoderma* sp. in a disc diffusion assay to assess activity (Fig. 4B; Fig. S5). The extracts from an isolate containing both compounds (ICBG1719) demonstrated inhibition against *Escovopsis weberi*, while extracts containing only one compound, either burkholdine or pyrrolnitrin (SID20365, pyrrolnitrin; SID20345, burkholdine1213), and the dimethyl sulfoxide (DMSO) control did not inhibit *Escovopsis weberi*. This reflected the results of the previous antimicrobial inhibition bioassays using *Burkholderia* isolates. Additionally, when the extracts from SID20365 (only pyrrolnitrin) and SID20345 (only burkholdine1213) were combined, creating an extract that artificially contained both compounds, inhibition was observed (Fig. 4C). This suggests that pyrrolnitrin and burkholdine1213 may act additively or synergistically to inhibit *Escovopsis* spp.

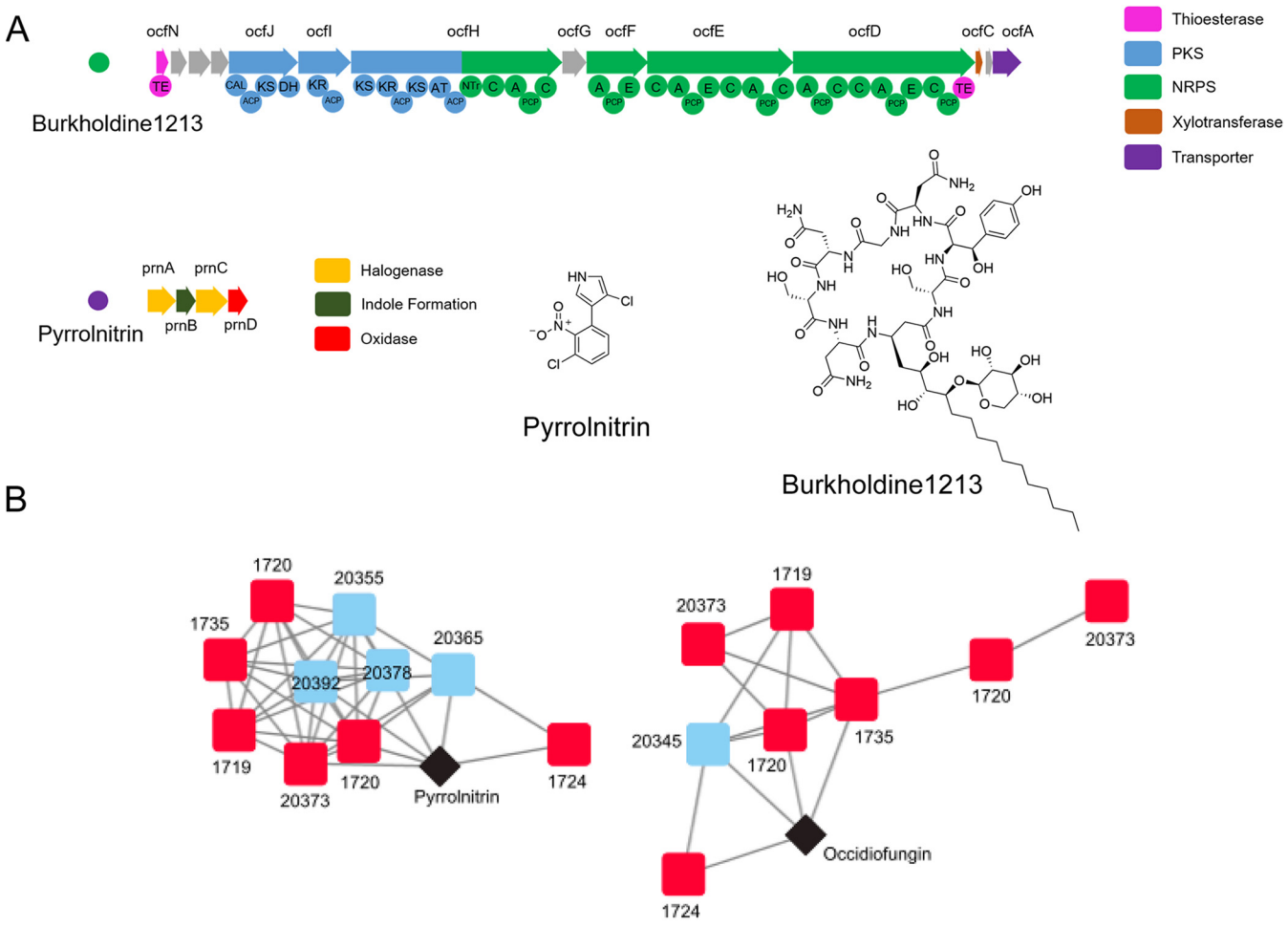

FIG 3 Identification of pyrrolnitrin and burkholdine1213 biosynthetic gene clusters in inhibitory isolates. (A) Genetic architecture of biosynthetic gene clusters encoding the production of burkholdine1213 and pyrrolnitrin identified by AntiSMASH 4.0. Domains of the PKS/NRPS hybrid biosynthetic gene cluster are shown beneath the gene representations for burkholdine1213. PKS, polyketide synthase; NRPS, nonribosomal peptide synthetase. (B) BiG-SCAPE network analysis of the two biosynthetic gene clusters present in *Burkholderia* isolates. Red squares are strong inhibitory *Burkholderia* isolates, blue squares are less inhibitory isolates, and black diamonds are database matches to the MIBiG biosynthetic gene cluster database.

***Burkholderia* extracts inhibit *Escovopsis weberi* growth at lower concentrations than those used to inhibit *Leucoagaricus* sp.** The lack of inhibition of other ecologically relevant fungi suggested that the antifungals produced by inhibitory *Burkholderia* isolates may be able to inhibit *Escovopsis* while not harming *Leucoagaricus*. To test this, six *Leucoagaricus* species strains and an *Escovopsis weberi* strain (CF180408-01) were grown individually on plates containing 0.005 mg/ml, 0.05 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2.5 mg/ml *Burkholderia* extract from an isolate with both pyrrolnitrin and burkholdine1213 (ICBG1719). All plates with extract concentrations above and including 0.5 mg/ml completely inhibited all growth of *Leucoagaricus* spp. and *Escovopsis weberi*. After 6 days, at 0.05 mg/ml, the growth rates (i.e., smaller diameters) observed for four out of the six *Leucoagaricus* spp. were lower than those of the control, while two *Leucoagaricus* spp. and *Escovopsis weberi* demonstrated no growth (Fig. S6) (Wilcoxon signed-rank test, $Z = -1.51$, $P = 0.1289$). Finally, at 0.005 mg/ml, the diameters of all *Leucoagaricus* strains grew comparably to the diameter of the control, while the growth of *Escovopsis weberi* was inhibited (Fig. 5) (Wilcoxon signed-rank test, $Z = -2.52$, $P = 0.0115$).

DISCUSSION

Here, we demonstrate that *Burkholderia* fungus garden isolates inhibit the parasitic fungus *Escovopsis in vitro*. The inhibition of *Escovopsis* spp. corresponds with the presence

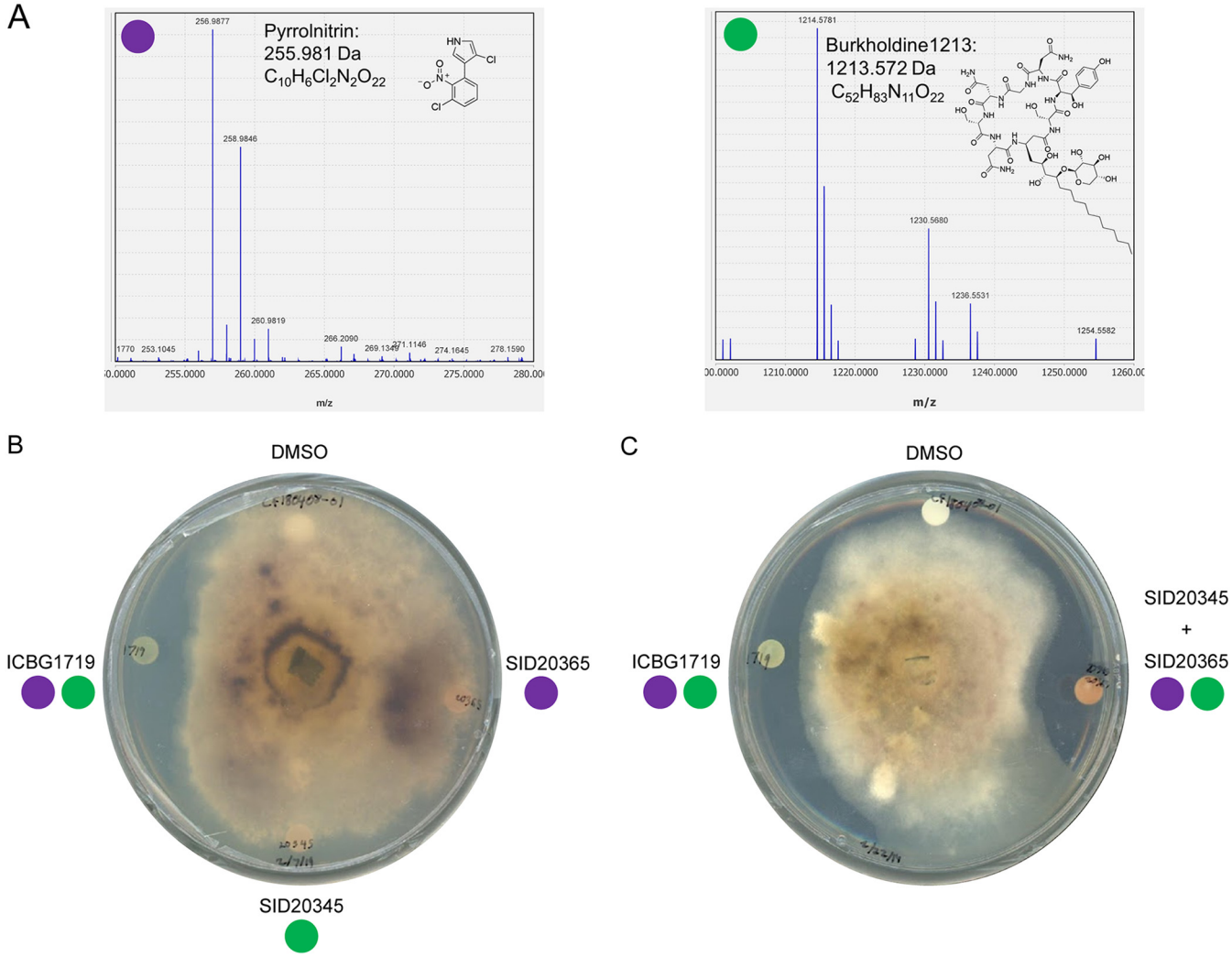

FIG 4 Detection of pyrrolnitrin and burkholdine1213 in organic extracts of inhibitory isolates and demonstration of extract activity. (A) Extracted ion chromatograms of *m/z* values that match those of pyrrolnitrin and burkholdine1213 from the organic extract of inhibitory *Burkholderia* isolate ICBG1719. (B) Disc diffusion assay of extracts from ICBG1719 (both pyrrolnitrin and burkholdine1213), SID20345 (burkholdine1213), and SID20365 (pyrrolnitrin) against *Escovopsis weberi* (CF180408-01). (C) Disc diffusion assay of extracts from ICBG1719 (both pyrrolnitrin and burkholdine1213) and a combined extract of SID20345 and SID20365 (artificially containing both pyrrolnitrin and burkholdine1213) against *Escovopsis weberi* (CF180408-01), demonstrating that both compounds must be present for inhibition. Additional disc diffusion assays were conducted on *Trichoderma*, *Aspergillus*, and *Fusarium* (Fig. S5).

of two BGCs that encode the production of two known antifungal compounds, pyrrolnitrin and burkholdine1213. We identified both antifungals in the extracts of inhibitory isolates, confirming the expression of these clusters when cultured. A combination of the extracts of isolates that contained only one or the other antifungal replicated the inhibitory activity, suggesting that both compounds must be present for anti-*Escovopsis* activity. Additionally, extracts of the inhibitory *Burkholderia* isolates were capable of inhibiting *Escovopsis weberi*, but not *Leucoagaricus* spp., at concentrations as low as 0.05 mg/ml *in vitro*. These findings suggest an important role for *Burkholderia* in the defense of fungus gardens from the parasitic fungus *Escovopsis* but not other ecologically relevant transient fungal invaders (e.g., *Trichoderma*).

Two lineages of fungus-growing ants, *Atta* and *Sericomyrmex*, have secondarily lost the ability to harbor the defensive symbiont *Pseudonocardia* (23). Four of the five strong inhibitory isolates were isolated from *Atta* colonies, and one isolate whose genome contained the BGC for pyrrolnitrin, SID20355, was isolated from a *Sericomyrmex* colony and had an average zone of inhibition of 1.7 cm in inhibition bioassays against the eight *Escovopsis* species strains (Fig. 2; Fig. S2). We sampled five colonies of *Sericomyrmex* and

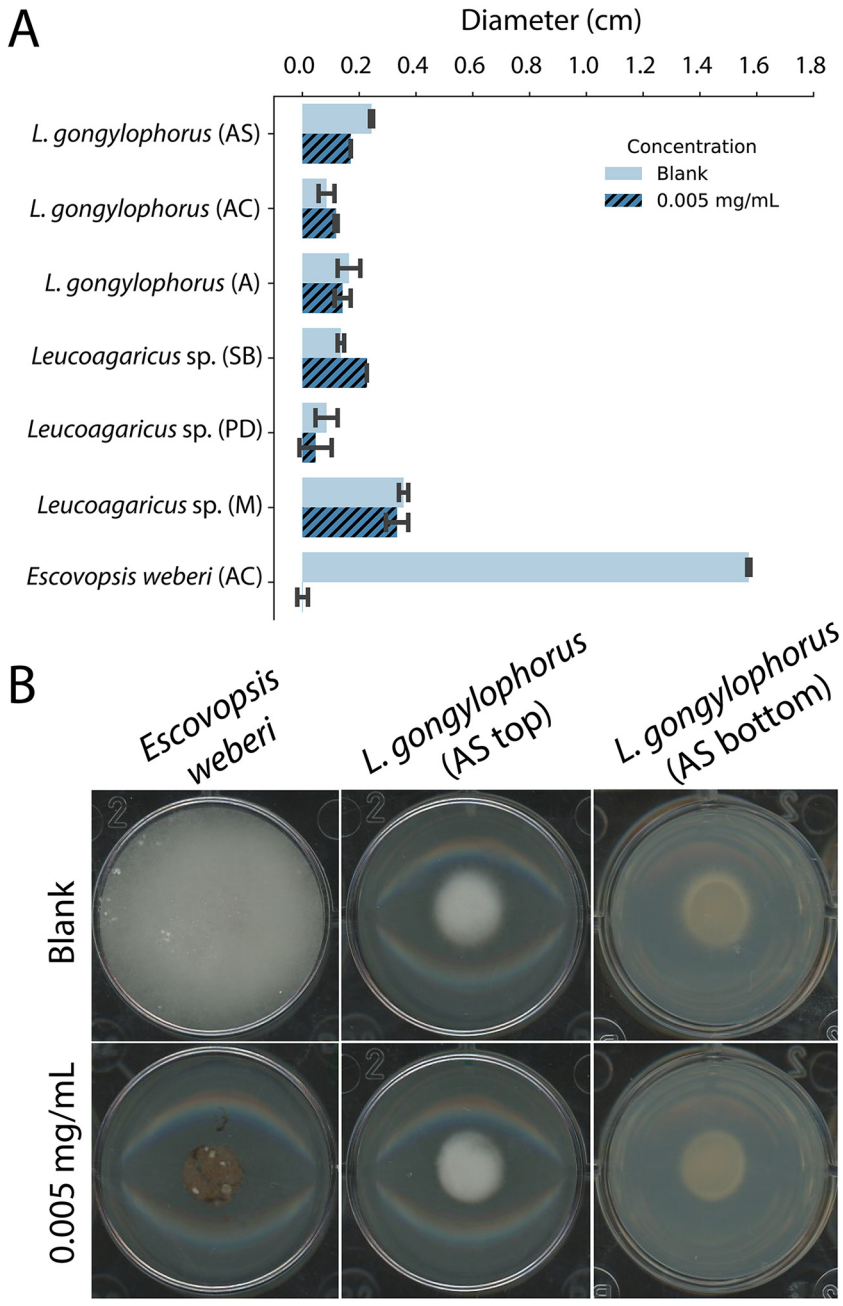

FIG 5 *Leucoagaricus* spp. grown on agar containing 0.005 mg/ml ICBG1719 extract (both BGCs) grow comparably to agar containing no extract, while *Escovopsis weberi* (CF180408-01) from an *Atta cephalotes* colony is inhibited. (A) The bar graph indicates the growth of *Leucoagaricus* spp. ($n = 2$ for each strain) and *Escovopsis weberi* ($n = 3$) after 6 days of growth on agar containing no extract (blank) or 0.005 mg/ml. For visual representation, the diameter of the fungal plug (0.6 mm) was subtracted from the overall diameter measurement. (B) Photographs represent typical fungal growth of *Escovopsis weberi* and *Leucoagaricus* spp. (photo is from *Leucoagaricus gongylophorus* from an *Atta sexdens* colony) on agar with no extract (blank) and 0.005 mg/ml extract. Photos include the top of the plate (AS top) and bottom of the plate (AS bottom). AS, *Atta sexdens*; AC, *Atta cephalotes*; A, *Acromyrmex* sp.; SB, *Sericomyrmex bondari*; PD, *Paratrachymyrmex diversus*; M, *Myrmicocrypta* sp.

were able to obtain at least one *Burkholderia* isolate from all five colonies, with a range of inhibition zones from 0.29 cm to 1.7 cm. Though we equally sampled between colonies in which *Pseudonocardia* was present and absent (26 fungus gardens from each group), there was uneven geographic sampling, and different bacterial isolation techniques and specific ant genus sampling may have influenced our results (Table 1). Our

results suggest *Burkholderia* species as potential defensive symbionts; however, additional work must be done to explore whether *Burkholderia* spp. are a potential replacement (37, 38) for *Pseudonocardia* in colonies without the symbiont or, more generally, if *Burkholderia* plays a larger role in defending the garden when *Pseudonocardia* is absent.

The presence of inhibitory *Burkholderia* and other known measures of sanitation within the fungus-growing ant system (25, 26) suggest that fungus-growing ants use multiple strategies to promote healthy fungus gardens. Of note, while we focused on strong inhibitory *Burkholderia* isolates containing two BGCs, some *Burkholderia* isolates from fungus gardens containing either one BGC or neither were still able to less severely inhibit *Escovopsis* spp. (Fig. 2; Fig. S2). This suggests that *Burkholderia* or other garden bacteria might play a role in inhibiting *Escovopsis* spp. in other fungus-growing ant genera. It appears unlikely that they play a role in the inhibition of other alien fungi, as we saw that *Burkholderia* isolates barely, if at all, inhibited *Trichoderma*, *Aspergillus*, and *Fusarium*. However, previous work with different fungus garden isolates has indicated the inhibition of other fungi, including *Trichoderma* (27, 39).

As with internal animal gut microbiomes, the fungus garden microbiome is composed of a complex and diverse microbial community where the roles of resident bacteria are still being established. In recent years, there has been an accumulation of evidence that members of the fungus garden microbiome potentially function as beneficial symbionts, with some taxa aiding in nitrogen fixation, degradation of plant matter, and/or detoxification of plant secondary compounds. In this study, we have described a fungus garden-associated bacterial genus, *Burkholderia*, as a possible mechanism for certain fungus-growing ant lineages to defend their fungus gardens from parasites. Overall, these new findings combined with past studies suggest that members of the fungus garden microbiome play a key role in facilitating the response of fungus-growing ants to environmental changes and pressures.

MATERIALS AND METHODS

Sampling and bacterial isolations. We collected fungus garden samples in January 2017 and April 2018 in the following locations: Anavilhanas, Amazonas, Brazil; Ducke Reserve, Amazonas, Brazil; Itatiaia, Rio de Janeiro, Brazil; Sao Paulo State, Brazil; and La Selva Biological Station, Costa Rica (see Data Set S1 in the supplemental material). Collections of biological samples and research on genetic resources were authorized in Brazil by SISBIO number 46555-5 and CNPq number 010936/2014-9. In Costa Rica, permits were granted by the Comisión Institucional de Biodiversidad of the University of Costa Rica (resolution number 009) and collections were authorized by the Organization of Tropical Studies (OTS), under UCR project 801-B9-515. For the fungus garden samples collected in Brazil, for each colony, a small fungus garden piece collected from the inner region of the garden was put into phosphate-buffered saline (PBS) and then plated onto yeast malt extract agar (YMEA). Plates with bacterial colonies were brought back to the University of Wisconsin—Madison, and pure bacterial isolates were obtained after several rounds of subculturing. We obtained a total of 317 bacterial isolates. We identified 117 isolates to the genus level by whole 16S rRNA gene sequencing. Of those, 33 corresponded to the genus *Burkholderia*. For the fungus garden samples collected in Costa Rica, ~0.2 g of fungus garden was collected from the inner region of the garden, put into PBS, vortexed, and homogenized; then the PBS-fungus garden homogenate was serially diluted, and all dilutions were plated on both YMEA and modified APCA (40) [per liter: 0.79 g $(NH_4)_2SO_4$, 1 g KH_2PO_4, 0.5 g $MgSO_4$-H_2O, 0.2 g KCl, 2 g L-arabinose, 5 mg crystal violet, 50 mg polymyxin B sulfate, 50 mg ampicillin sodium, 10 mg chloramphenicol, 15 g agar].

DNA extraction and assembly. We extracted DNA from 30 bacterial isolates using the Wizard genomic DNA purification kit (Promega, USA) using the Gram-negative protocol. Genomic DNA libraries for Illumina MiSeq 2× 300-bp paired-end sequencing were prepared by the University of Wisconsin—Madison Biotechnology Center. Reads were corrected with MUSKET v1.1 (41), and paired-ends were merged with FLASH v.1.2.7 (42) and assembled with SPAdes 3.11.0 (43). We taxonomically classified the *Burkholderiaceae* isolates to the species level by performing a tetra correlation search at JSpeciesWS (44). In addition, ANI was calculated using the anvi'o (45) anvi-compute-genome-similarity program, with the default pyani (46) settings.

Phylogenetic tree. We generated a genome-based, multilocus *Burkholderiaceae* phylogeny based on previous methods (47). Briefly, the phylogeny was generated using 93 full TIGRFAM proteins in the "core bacterial protein" set (GenProp0799) as the molecular data set. The protein sequences with the top HMMER bit score for each protein family were aligned using MAFFT (48) and were then converted to codon alignments and concatenated. RAxML-7.2.6 (49) was used to generate the phylogeny using the GTRgamma substitution model and 100 rapid bootstraps on the final, recombination-free alignment. The gene tree-based phylogeny was generated using ASTRAL-II (50). Phylogenies were visualized and edited in FigTree v1.4.3.A. We constructed the 16S rRNA gene phylogenetic tree using BEAST2 v2.5.1 (51) with a TN93 substitution model. The analysis was run for 150,000,000 generations, with every 1,000

generations sampled, and a burn-in of 10% was applied. The code used for this analysis can be accessed at https://github.com/chevrm/core_species_tree.

***Escovopsis-Burkholderiaceae* bioassays.** In order to assess inhibition profiles of *Burkholderiaceae* against *Escovopsis* and other fungi, we performed *in vitro* plate assays. For each isolate, we grew an overnight culture in yeast malt extract (YME) for 16 to 24 h. We spotted 10 μl of *Burkholderiaceae* onto the middle of a 100-mm potato-dextrose agar (PDA) plate, placed Parafilm on the plates, and incubated them at room temperature for 7 days. After 7 days, 6-mm fungal plugs of *Escovopsis*, *Trichoderma*, *Aspergillus*, and *Fusarium* were put on the edge of the plate. Control plates containing only a *Burkholderiaceae* isolate or only a fungus were also made for each isolate. All plates were covered with two pieces of Parafilm. Over the course of a month, both sides of the plate were scanned every 6 to 7 days, and the zone of inhibition was measured using Fiji (52). The eight *Escovopsis* strains were chosen because they were isolated from different lineages of fungus-growing ants (*Acromyrmex*, *Apterostigma*, *Atta*, *Cyphomyrmex*, *Myrmicocrypta*) (Data Set S1). *Escovopsis* species IDs are given in Data Set S1. *Trichoderma* sp., *Aspergillus flavus*, and *Fusarium oxysporum* are ecologically relevant fungi and represent different degrees of relatedness to *Escovopsis* spp.

Biosynthetic gene cluster annotation. We used antiSMASH v4.0 (53) to predict BGCs and BiG-SCAPE (54) to construct sequence similarity networks of BGCs. In addition to including our predicted BGCs for the BiG-SCAPE analysis, we included the reference BGCs from the MIBiG repository. We used cytoscape (55) to visualize the BGC networks. We color coded the clusters into two categories: strong inhibitory *Burkholderia* isolates (average ZOI $\geq$ 2.45 cm against *Escovopsis*) and noninhibitory *Burkholderia* isolates (average ZOI $\leq$ 2.45 cm against *Escovopsis*) and looked for clusters that contained all the inhibitory isolates. We subsequently used Clinker (56) and BLASTP to determine the similarity of the two antifungal-encoding BGCs from the *Burkholderia* isolates to the characterized BGCs from the MIBiG repository.

***Burkholderia* extracts.** *Burkholderia* isolates ICBG1719, ICBG1720, ICBG1724, ICGB1735, SID20373, SID20345, SID20365, and ICBG849 were inoculated from YMEA plates into 100 ml of YME broth in 500-ml baffled flasks and shaken for 36 h. The 100-ml cultures were used to inoculate two 500-ml cultures of YME broth in 2-liter flasks for each isolate. The isolates were shaken for 36 h with Diaion HP20 resin. The cultures were then vacuum filtered through a Whatman 1-sized filter paper, and the Diaion resin and cell mass was extracted overnight with ethyl acetate. Excess anhydrous sodium sulfate was added to the extraction to remove residual water. The ethyl acetate was filtered and dried *in vacuo* to yield a *Burkholderia* extract.

HRESIMS of *Burkholderia* extracts. *Burkholderia* extracts were resuspended in methanol and analyzed for the presence of pyrrolnitrin and burkholdine1213 by UPLC-HRESIMS on a Q Exactive Orbitrap mass spectrometer. A liquid chromatography gradient was run from 5% acetonitrile with 0.1% formic acid to 100% acetonitrile with 0.1% formic acid over 15 min on a Phenomenex XB C_{18}, 2.1-mm by 100-mm, 2.6-μm-particle-size column. The scan range was from 200 *m/z* to 2,000 *m/z* in positive mode.

***Escovopsis-Burkholderia* extract assays.** *Escovopsis clavatus* (ICBG1053, *Apterostigma*) and *Escovopsis weberi* (CF180408-01, *Atta cephalotes*) plugs were plated onto PDA and grown for 3 days until white mycelia could be seen. We chose these two *Escovopsis* strains because they were representative of the *Escovopsis-Burkholderia* inhibition profiles. Additionally, *Trichoderma*, *Aspergillus*, and *Fusarium* were plated on PDA and grown until slight mycelial growth was visible. *Burkholderia* extracts from ICBG1719, SID20345, and SID20365 were dissolved in dimethyl sulfoxide (DMSO). The extracts were pipetted onto sterile filter paper discs in 10 μl of DMSO at 0.5 mg/disc, 1 mg/disc, and 2 mg/disc and placed onto the PDA plates containing the *Escovopsis* or other fungi along with a DMSO control disc. After 2 weeks of growth, the ability of each extract to inhibit the growth of the fungi was assessed and pictures were taken of the plates.

***Leucoagaricus-Burkholderia* extract assays.** We plated six *Leucoagaricus* strains isolated from *Atta sexdens*, *Atta cephalotes*, *Acromyrmex octospinosus*, *Sericomyrmex bondari*, *Paratrachymyrmex diversus*, *Myrmicocrypta* sp., and an *Escovopsis weberi* isolate (CF180408-01) from *Atta cephalotes* on PDA and let them grow for a month at room temperature. We prepared PDA plates containing 0 mg/ml, 0.005 mg/ml, 0.05 mg/ml, 0.5 mg/ml, 1 mg/ml, and 2.5 mg/ml *Burkholderia* extract in DMSO. Media were vigorously mixed for homogenous distribution of the extract, and then 3 ml was pipetted into each well of a 12-well plate (catalog no. 82050-926; Greiner Bio-One) and left to dry overnight in the dark. Then, 0.6-mm plugs taken from the outer edges of *Leucoagaricus* species or *Escovopsis* fungal plates were deposited into the center of each well ($n = 2$ for each fungal strain at 6 concentrations). Pictures were taken 6 days postexposure, and the diameter of fungal growth was measured in Fiji. To test if *Leucoagaricus* species growth was significantly greater than *Escovopsis weberi* growth with 0.05 mg/ml and 0.005 mg/ml *Burkholderia* extract, we performed a Wilcoxon signed-rank test in JMP Pro 14 using the average growth of all *Leucoagaricus* species strains ($n = 2$ replicates $\times$ 6 strains = 12) and the average growth of *Escovopsis weberi* ($n = 3$ replicates $\times$ 1 strain = 3) for each concentration.

Data availability. All sequencing data have been uploaded into the NCBI databases under BioProject numbers PRJNA564151 and PRJNA603049. Whole-genome and SRA accession numbers for each isolate can be found in Data Set S2. Whole 16S rRNA gene sequences are under the GenBank accession numbers MW756842 to MW756915 and MW772240 to MW772251. Data Set S1 includes individual accession numbers.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

SUPPLEMENTAL FILE 1, XLSX file, 0.02 MB.

SUPPLEMENTAL FILE 2, XLSX file, 0.01 MB.

SUPPLEMENTAL FILE 3, XLSX file, 0.2 MB.
SUPPLEMENTAL FILE 4, PDF file, 4.1 MB.

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