Generalized antifungal activity and 454-screening of *Pseudonocardia* and *Amycolatopsis* bacteria in nests of fungus-growing ants

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In many host-microbe mutualisms, hosts use beneficial metabolites supplied by microbial symbionts. Fungus-growing (attine) ants are thought to form such a mutualism with *Pseudonocardia* bacteria to derive antibiotics that specifically suppress the coevolving pathogen *Escovopsis*, which infects the ants' fungal gardens and reduces growth. Here we test 4 key assumptions of this *Pseudonocardia-Escovopsis* coevolution model. Culture-dependent and culture-independent (tag-encoded 454-pyrosequencing) surveys reveal that several *Pseudonocardia* species and occasionally *Amycolatopsis* (a close relative of *Pseudonocardia*) co-occur on workers from a single nest, contradicting the assumption of a single pseudonocardiaceous strain per nest. *Pseudonocardia* can occur on males, suggesting that *Pseudonocardia* could also be horizontally transmitted during mating. *Pseudonocardia* and *Amycolatopsis* secretions kill or strongly suppress ant-cultivated fungi, contradicting the previous finding of a growth-enhancing effect of *Pseudonocardia* on the cultivars. Attine ants therefore may harm their own cultivar if they apply pseudonocardiaceous secretions to actively growing gardens. *Pseudonocardia* and *Amycolatopsis* isolates also show nonspecific antifungal activities against saprotrophic, endophytic, entomopathogenic, and garden-pathogenic fungi, contrary to the original report of specific antibiosis against *Escovopsis* alone. We conclude that attine-associated pseudonocardiaceous bacteria do not exhibit derived antibiotic properties to specifically suppress *Escovopsis*. We evaluate hypotheses on nonadaptive and adaptive functions of attine integumental bacteria, and develop an alternate conceptual framework to replace the prevailing *Pseudonocardia-Escovopsis* coevolution model. If association with *Pseudonocardia* is adaptive to attine ants, alternate roles of such microbes could include the protection of ants or sanitation of the nest.

mutualism | symbiosis | Attini | Actinomycete | *Escovopsis*

Gardens of fungus-growing ants (Attini, Formicidae) are complex communities of microbes. The living biomass of an attine garden is dominated by a monoculture of basidiomycete fungus that is tended by the ants as food (1), but additional microbes such as filamentous fungi, yeasts, and bacteria grow alongside the cultivated fungus in the garden matrix, as well as on the ants themselves. These secondary microbes interact in antagonistic, commensal, or mutualistic ways with each other, with the cultivated fungus, and with the host ants (1–8).

A diversity of nonmutualistic "weed" fungi are known to grow in attine gardens, such as microfungi in the genera *Trichoderma*, *Fusarium*, or *Syncephalastrum* (1, 6, 7, 9, 10) but the best-studied fungal invaders in attine gardens are filamentous, ascomycetous fungi in the genus *Escovopsis* (Hypocreaceae, Hypocreales) (9). Because of an ability to parasitize cultivar mycelium (11), *Escovopsis* can devastate an entire garden (9). Attine ants have evolved defenses against such diseases, such as physical weeding, antibiotic secretion, and management of disease-suppressing auxiliary microbes (1, 4, 5). The most prominent microbes thought to be involved in disease-suppression in attine gardens are actinomycete bacteria in the genus *Pseudonocardia*, which accumulate on the ants' bodies mixed into integumental accretions of likely glandular origin (12–14). Many of the ant-associated *Pseudonocardia* species show antibiotic activity in vitro against *Escovopsis* (13–15). A diversity of actinomycete bacteria including *Pseudonocardia* also occur in the ant gardens, in the soil surrounding attine nests, and possibly in the substrate used by the ants for fungiculture (16, 17).

The prevailing view of attine actinomycete-*Escovopsis* antagonism is a coevolutionary arms race between antibiotic-producing *Pseudonocardia* and *Escovopsis* parasites (5, 18–22). Attine ants are thought to use their integumental actinomycetes to specifically combat *Escovopsis* parasites, which fail to evolve effective resistance against *Pseudonocardia* because of some unknown disadvantage in the coevolutionary arms race (14, 18, 20). This view on specific *Pseudonocardia-Escovopsis* coevolution was based on very little direct evidence in support of 4 key observations. First, in 2 species studied so far using PCR-based bacterial screens (with *Pseudonocardia*-specific primers), workers of a single attine nest were thought to associate with only one *Pseudonocardia* lineage (23). Second, in 2 species studied so far for presence/absence of bacterial growth on reproductives, attine queens carried visible growth during their mating flights, but not the males, suggesting vertical transmission from mother to daughter queen (18); this is expected to generate selection for beneficial bacterial traits within a long-term ant-*Pseudonocardia* partnership (5, 18, 20, 24). Third, one study showed that a single, unidentified actinomycete bacterium isolated from an *Apterostigma* worker secreted compounds that enhanced the growth of the cultivated fungus, suggesting a derived actinomycete metabolism promoting the ant-cultivar mutualism (18). Fourth, a single study involving a single *Pseudonocardia* strain isolated from an *Acromyrmex* worker showed that this particular bacterium secreted antibiotics with specific activity targeting *Escovopsis* but no activity against 17 other test fungi, suggesting an evolutionarily derived state of specific antibiosis (18), rather than generalized antibiosis typical for actinomycete bacteria at large (25, 26).

Here we present microbiological and antibiotic evidence that contradict each of the above observations, adding to recent phylogenetic evidence that questioned the plausibility of *Pseudonocardia-Escovopsis* coevolution (17). Most importantly, *Pseudonocardia* of various attine species have nonspecific antibiotic properties that inhibit garden pathogens, endophytes, saprotrophs, arthropod pathogens, and most severely the ant-cultivated fungi. We evaluate hypotheses on nonadaptive and adaptive functions of attine integumental bacteria and develop an alternative conceptual framework

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Table 1. Actinomycete morphotypes and their respective attine sources, isolated with culture-dependent methods (W = worker; G = garden; M = male; F = winged female)

	Code	Color of Medium	Color of Bacterial Colony	Growth Form of Bacterial Colony	*Cypho. wheeleri* 2701	*Trachy. turrifex* 2701	*Trachy. septen-trionalis* 0803	*Trachy. zeteki* 1812	*Trachy. zeteki* 1603	*Myco. smithii* 0208	*Myco. smithii* 1701	*Myco. smithii* 2930	*Myco. smithii* 2902	*Myco. smithii* 0103	*Serico amabilis* 0423	Genus (group*)	Genbank Accession	Photo Number
1	BMWB1	Dark brown	White-brown	Rosette						W		W	W	W		*Pseudonocardia* (*compacta*-group)	FJ948119	S3
2	T1	Transparent	Light brown center, white periphery	Rosette	W,M,F						W	W, G		W	W, M	*Pseudonocardia* (*compacta*-group)	FJ948116, FJ948117	S3
3	TMWB1	Transparent	White	Rosette button big							W			W		*Pseudonocardia* (*thermophila*-group)	FJ948118	S3
4	GMWB1	Dark green	White-cream	Small button		W, M										*Pseudonocardia* (*alni*-group)	FJ948120, FJ948129	S3
5	Y1	Transparent	Bright yellow	Rosette	W,M											*Pseudonocardia* (*thermophila*-group)	FJ948115	S3
6	TMBB1	Transparent	Brown	Small button			W									*Pseudonocardia* (*alni*-group)	FJ948121	S3
7	Amy1	Yellowish pink	Whitish pink	Diffused with halo						W		W	W	W		*Amycolatopsis sp.*	FJ948128	S3
8	Actino3	Light yellow	White	Diffused with halo						W						*Pseudonocardia* (*thermophila*-group)	FJ948122	S2
9	TMWB2	Transparent	White front, yellow back	Medium-sized button							W			W		*Pseudonocardia.* (*thermophila*-group)	FJ948123	S3
10	T4/T10	Transparent	White front, yellow back	Diffused with halo										W		*Streptomyces sp.*	FJ948124	S3
11	TMYB1	Transparent	Light yellow	Big rosette							W					*Pseudonocardia* (*thermophila*-group)	FJ948125	S3
12	TMWB3	Transparent	White	Small button				W	W						M	*Pseudonocardia* (*alni*-group)	FJ948126, FJ948127	S3

**Pseudonocardia* groups are assigned according to phylogenetic clades provisionally defined in Mueller et al. (17).

to replace the prevailing *Pseudonocardia-Escovopsis* model (18–24, 27). For those attine ants for which association with *Pseudonocardia* is adaptive, possible alternate roles of the integumental bacteria could include protection of the ants, control of cultivar growth, or sanitation of the nest environment.

Results

Diverse Pseudonocardiaceous Bacteria in Single Attine Nests. All ant and garden samples surveyed with culture-independent 454-pyrosequencing (total of 41,561 16S-amplicon sequences) contained several *Pseudonocardia* species and other pseudonocardiaceous species, as well as a great diversity of additional actinomycete species (e.g., *Gordonia*, *Microlunatus*, *Mycobacterium*) (Tables S1 and S2; Figs. S1 and S2; *S1 Results*). For example, on *Trachymyrmex septentrionalis* workers from the same nest, 2 *Pseudonocardia* species (*P. cf. spinosispora* and *P. cf. ammonioxydans*) co-occurred in comparable proportions (Table S1). These 2 *Pseudonocardia* species, which belong to 2 distinct subgroups of *Pseudonocardia* (17), comprised almost 100% of all pseudonocardiaceous species, 60% of all actinomycetes, and 4.7% of all bacteria characterized for *T. septentrionalis* workers. In contrast, no such predominance of only 2 *Pseudonocardia* lineages occurred in the bacterial communities of *Mycocepurus smithii* and *Cyphomyrmex wheeleri* workers, where several distantly related *Pseudonocardia* species and other pseudonocardiaceous genera (e.g., *Amycolatopsis*) coexisted in significant abundance on workers (Table S1).

Unlike the culture-independent 454-screen, culture-dependent isolations revealed only a single species of *Pseudonocardia* in each *Trachymyrmex* nest ($n = 3$ nests), but confirmed the coexistence of several *Pseudonocardia* species in each of the 5 *M. smithii* nests and the single *C. wheeleri* nest (Table 1, Fig. S3). The diversity difference in the case of *Trachymyrmex* reflects the well-known observation that culture-dependent methods generally underestimate microbial diversities. Workers of *C. wheeleri* carried significant abundances of 2 distantly related *Pseudonocardia* species, and workers of *M. smithii* carried between 2–4 *Pseudonocardia* species in addition to one species of *Amycolatopsis* (Table 1). In 3 out of 5 *M. smithii* nests, *Pseudonocardia* colony-forming-units (CFUs) were more abundant than *Amycolatopsis* CFUs (Wilcoxon sign-rank test, $P < 0.02$; see *SI Results*); whereas in one nest, CFUs of *Pseudonocardia* and *Amycolatopsis* were not significantly different (bacteria abundance could not be counted in the fifth nest because of fungal growth on the plate). While we could readily isolate pseudonocardiaceous bacteria from *M. smithii*, *C. wheeleri*, *Trachymyrmex zeteki*, *Trachymyrmex turrifex*, *T. septentrionalis*, and *Sericomyrmex amabilis*, we failed to find *Pseudonocardia* on workers or in gardens of the leafcutter ants *Atta texana*, *Atta sexdens*, and *Acromyrmex coronatus* with the culture-dependent method. We found traces of *Pseudonocardia* in the *Atta cephalotes* garden, but not a single *Pseudonocardia* on workers of the same nest.

Presence of *Pseudonocardia* on Males. We could isolate 2 species of distantly-related *Pseudonocardia* from *C. wheeleri* males (Table 1). These 2 *Pseudonocardia* species were also found in workers from

Table 2. Growth responses of the test fungi challenged with different *Pseudonocardia* and *Amycolatopsis* isolates (NG = no growth; AG = attenuated growth; TB = bacteria touch growth; FG = Full growth; – = not tested). See Table S3 for sources and codes of bacteria and Table S4 for sources of fungi

		Pseudonocardia										*Amycolatopsis*	
Type of fungus	Species name (Test code)	P1	P2	P3	P4	P5	PY1	PT1 (Cwh)	PT1 (Msm)	P TM WB1	P BMWB1	Amy1	Amy2
Cultivar	*Leucocoprinus* sp. (Test 22)	AG	AG	AG	AG	AG	AG	NG	NG	NG	AG	NG	NG
Cultivar	*Leucocoprinus* sp. (Test 6)	NG	NG	NG	AG	AG	AG	AG	NG	NG	AG	NG	NG
Cultivar	*Leucocoprinus* sp. (Test 8)	NG	NG	AG	AG	AG	–	–	AG	NG	AG	AG	NG
Cultivar	*Leucocoprinus* sp. (Test 9)	NG	NG	AG	AG	AG	NG	NG	AG	NG	AG	NG	NG
Cultivar	*Leucocoprinus* sp. (Test 11)	NG	NG	AG	AG	AG	AG	AG	NG	NG	AG	NG	NG
Cultivar	*Leucocoprinus* sp. (Test 13)	NG	NG	AG	AG	AG	AG	TB	–	–	–	NG	NG
Cultivar	*Leucocoprinus* sp. (Test 15)	NG	NG	NG	AG	NG	AG	NG	NG	NG	NG	NG	NG
Entomopathogen	*Fusarium solani* (Test 4)	TB	TB	TB	TB	TB	TB	TB	AG	TB	TB	TB	TB
Entomopathogen	*Acrodontium* sp. (Test 16)	AG	AG	AG	AG	AG	NG	NG	–	–	–	AG	AG
Entomopathogen	*Beauveria bassiana* (3288)	FG	TB	FG	FG	FG	–	–	–	–	–	TB	FG
Entomopathogen	*Metarhizium anisopliae* (2575)	FG	AG	FG	FG	TB	–	–	FG	TB	TB	TB	TB
Entomopathogen	*Beauveria bassiana* (5465)	AG	AG	FG	FG	TB	AG	AG	TB	AG	FG	TB	AG
Entomopathogen	*Beauveria bassiana* (5991)	TB	TB	TB	TB	TB	–	–	–	–	–	TB	TB
Entomopathogen	*Beauveria bassiana* (6147)	TB	TB	FG	FG	TB	–	–	TB	TB	AG	TB	TB
Entomopathogen	*Beauveria bassiana* (6907)	–	–	–	–	–	–	–	FG	FG	TB	–	–
Endophyte/Entomopathogen	*Verticillium leptobactrum* (Test 17)	TB	TB	TB	AG	TB	–	–	TB	AG	AG	TB	TB
Endophyte	*Phoma* sp. (Test 27)	AG	AG	TB	AG	AG	–	–	–	–	–	AG	AG
Endophyte/Saprotroph	*Alternaria tenuissima* (Test 19)	FG	FG	FG	AG	FG	NG	NG	–	–	–	FG	FG
Saprotroph	*Cyphellophora* sp. (Test 5)	AG	AG	AG	TB	AG	TB	TB	TB	TB	AG	AG	AG
Garden pathogen	*Syncephalastrum racemosum* (Test 1)	AG	AG	TB	AG	TB	TB	TB	AG	–	–	AG	AG
Garden pathogen	*Escovopsis* sp. (Test 2)	AG	AG	AG	NG	NG	FG	TB	–	AG	AG	AG	AG
Garden pathogen	*Escovopsis* sp. (Test 23)	AG	AG	AG	AG	AG	–	–	NG	NG	TB	NG	NG
Garden pathogen	*Escovopsis* sp. (Test 25)	FG	AG	AG	FG	AG	FG	FG	TB	AG	FG	AG	AG
% Touch-bacteria Growth (TB)		18.2	22.7	22.7	13.6	31.8	21.4	35.7	31.3	25.0	25.0	31.8	22.7
% Full Growth (FG)		18.2	4.6	22.7	22.7	9.1	14.3	7.1	12.5	6.3	12.5	4.6	9.1
% Attenuated Growth (AG)		36.4	45.5	45.5	59.1	50.0	42.9	21.4	25.0	25.0	56.3	31.8	31.8
% No Growth (NG)		27.3	27.3	9.1	4.6	9.1	21.4	35.7	31.3	43.8	6.3	31.8	36.4
% Inhibition (NG & AG)		63.6	72.7	54.6	63.6	59.1	64.3	57.1	56.3	68.8	62.5	63.6	68.2

the same nest; however, only one type was found in the reproductive females with the culture-dependent method. We found 2 species of distantly-related *Pseudonocardia* in *S. amabilis* males and one of them in their nestmate workers. We could also isolate *Pseudonocardia* from *T. turrifex* males and the same *Pseudonocardia* strain from their nestmate workers (Table 1). Unfortunately, we could test for the presence of actinomycetes on males only in 3 attine species because other nests did not have males.

Nonspecific Antifungal Activity of *Pseudonocardia* and *Amycolatopsis*. All *Pseudonocardia* and *Amycolatopsis* isolates inhibited more than 50% (range 56.3–72.7%) of the test-fungi (Table 2, Fig. S5). Of the various test-fungi challenged (ant-cultivated fungi, saprotrophs, endophytes, entomopathogens, and garden-pathogens including *Escovopsis*), the pseudonocardiaceous secretions inhibited the ant-cultivated fungi most severely (Table 2, Fig. S4). Although we challenged the test-fungi at lower antibiotic concentrations than previous researchers (13, 14) (earlier work allowed accumulation of bacterial secretions for 3 weeks before testing, we allowed only for 2 weeks), 56.1% of the ant-cultivated fungi died when exposed to pseudonocardiaceous antibiotics. Out of 7 *Pseudonocardia* x cultivar combinations from natural nests, 4 cultivars showed no growth and 3 showed attenuated growth when challenged with *Pseudonocardia* isolated from the nests of their origin. *Escovopsis* was inhibited, but not always (Table 2). In some actinomycete-*Escovopsis* interactions, *Escovopsis* grew preferentially toward the actinomycete, encircled it (or grew over the actinomycete), then stopped growing (Fig. S4). We rarely observed the complete inhibition of *Escovopsis* reported previously (18). Both control and challenged *Escovopsis* exhibited a short period of rapid mycelial expansion; however, while actinomycete-challenged *Escovopsis* produced thin mycelial growth, followed by growth stagnation and occasional mycelial decay, control *Escovopsis* eventually produced a dense mycelium covering the entire test plate. In sum, all tested *Pseudonocardia* and *Amycolatopsis* from attine workers showed nonspecific activity affecting diverse fungi, but the ant-cultivated fungi were most severely inhibited by pseudonocardiaceous secretions.

Discussion

Workers of a Single Nest May Carry Several Pseudonocardiaceous Bacteria. We isolated multiple, phylogenetically diverse *Pseudonocardia* species from attine workers of the same nest (in *M. smithii* and *C. wheeleri*). In addition, culture-independent 454-screens established the coexistence of several *Pseudonocardia* species and additional pseudonocardiaceous lineages in workers from the same nest in the ant species surveyed with this technique (*T. septentrionalis*, *M. smithii*, *C. wheeleri*). Surprisingly, *M. smithii* workers carried abundant *Amycolatopsis* in addition to *Pseudonocardia*. While *Pseudonocardia* and *Amycolatopsis* lineages may not necessarily share the same nutritional niche on ants because these bacterial lineages are somewhat diverged, the coexistence of several *Pseudonocardia* species on a common nutrient pool supplied by the ants could lead to bacterial competition for resources (20, 28), suggesting that these bacteria could also evolve traits that confer advantages in bacteria–bacteria competition, but coincidentally harm the ants or their fungi. Indeed, we show that all pseudonocardiaceous bacteria inhibit a great diversity of fungi, but most strongly suppress or even kill the ant-cultivated fungi.

Nonspecific Antifungal Activity of *Pseudonocardia* and *Amycolatopsis*. Specialized activity of attine integumental *Pseudonocardia* only against *Escovopsis* (18) has been cited widely as evidence for

Pseudonocardia-Escovopsis coevolution (1, 5, 19, 21, 22, 27, 29–33). However, Sánchez-Peña et al. (34) and Oh et al. (35) recently showed that attine actinomycetes inhibit endophytic fungi and *Candida* yeasts. In addition, Kost et al. (36) showed that unidentified actinomycetes isolated from both attine and nonattine ants have comparable antibiotic activities. Our comprehensive screen of identified *Pseudonocardia* and *Amycolatopsis* isolated from attine workers now establishes (*a*) nonspecific activities of pseudonocardiaceous associates against a large array of problem fungi in attine nests (e.g., saprotrophs, entomopathogens), and (*b*) occasional attraction of *Escovopsis* to grow toward *Pseudonocardia*, rather than inhibition. *Pseudonocardia* associated with attine ants therefore do not secrete the evolutionarily derived, specific antibiotics predicted by the prevailing ant-*Pseudonocardia*-*Escovopsis* coevolution model (13, 14, 18, 20, 22, 24).

Ant-Associated *Pseudonocardia* and *Amycolatopsis* Can Harm the Cultivated Fungi. Currie et al. (18) tested a single, unidentified actinomycete strain isolated from *Apterostigma* ants and found a growth-enhancing effect on the corresponding cultivar. The stimulating effect of *Pseudonocardia* on cultivar growth has never been retested, but all of our tested cultivars were strongly suppressed or killed by *Pseudonocardia* and *Amycolatopsis* secretions isolated from workers of the corresponding nests. The ants would therefore harm or kill their own cultivar if they apply such secretions to their garden. Together with the findings of nonspecific antibiotic activity of *Pseudonocardia* and the frequent ineffectiveness against *Escovopsis*, the observation of severe cultivar inhibition could indicate that (*a*) *Pseudonocardia* is not used by the ants to sanitize gardens but serves some unknown function, or (*b*) the antibiotic effects on *Escovopsis* are merely a coincidental byproduct of these other functions, or (*c*) *Pseudonocardia* may actually be pathogenic rather than mutualistic. The latter interpretation is consistent with the observation that *Pseudonocardia* accretion causes metabolic stress in ants (16) but is less compatible with the observations that *Pseudonocardia* in some derived attine lineages occur preferentially on specific cuticular structures of the ants (14) and that some attine ants seem to be able to up-regulate *Pseudonocardia* abundance when a nest is experimentally infected with *Escovopsis* (37).

To minimize the potential damage to gardens, it is possible that the ants selectively apply pseudonocardiaceous secretions only locally to critically infected garden sections. In addition, the ants may apply secretions at concentrations lower than the concentrations tested in our and in previous in vitro experiments (14, 18, 20). In vivo, perhaps lower antibiotic concentrations suppress *Escovopsis* but do not harm the cultivars, but it is also possible that both the cultivar and *Escovopsis* are unaffected at low concentrations. Although we tested at concentrations lower than previous researchers, these latter possibilities weaken the significance of our antibiotic experiments, as well as the significance of previous antibiotic work on attine actinomycetes (13, 14, 18, 20, 24, 35, 38, 39). Future research will need to measure actual concentrations of ant-applied pseudonocardiaceous secretions in attine gardens and understand dose-dependent suppression of *Escovopsis*, cultivar, and other problem microbes.

Presence of *Pseudonocardia* on Attine Males. Significant levels of *Pseudonocardia* occurred on males of *C. wheeleri*, *T. turrifex*, and *S. amabilis*. Because the males carried the same *Pseudonocardia* species as their nestmate workers, it appears that males are colonized by bacteria derived from their nestmate workers or from a common source (e.g., garden, soil). Although it is possible that males carry lower bacterial loads in field nests, male mates now emerge as a potential vector for horizontal *Pseudonocardia* transfer between female lineages. In addition to frequent de novo acquisition from environmental sources (17, 30, 36), vectoring by males between female lineages can help explain why ant-*Pseudonocardia* associations are ephemeral over ecological time (40).

Amycolatopsis. *Amycolatopsis* isolates have similar or stronger antibiotic properties to *Pseudonocardia* (Fig. S5). None of the previous studies reported *Amycolatopsis* from attine ants, except for *Amycolatopsis* sequences in PCR screens of *T. turrifex* (17). Several reasons can explain the general absence in previous reports, including incompleteness of previous culture-dependent screens and methodological differences (see *SI Results*). While the presence of *Amycolatopsis* is intriguing because this genus produces well-known pharmaceuticals (rifampicin, vancomycin), further study will need to characterize the nature of the *Mycocepurus-Amycolatopsis* association.

A Reevaluation of the Attine Ant-Actinomycete Symbiosis. We fail to confirm key assumptions of the prevailing ant-*Pseudonocardia-Escovopsis* model of coevolution. First, more than one *Pseudonocardia* species and sometimes the closely related *Amycolatopsis* can co-occur abundantly on workers of the same nest; and second, *Pseudonocardia* on workers are not specialized to inhibit *Escovopsis.* Together with the recent realization that *Pseudonocardia* probably frequently colonize attine ants from environmental sources (17, 36, 40), our findings overturn the prevailing view that *Pseudonocardia* are obligate mutualistic associates supplying the ants with antibiotics to specifically suppress *Escovopsis*. Alternate interpretations—that *Pseudonocardia* are mutualists serving unknown purposes, or are commensal or pathogenic associates—now appear also plausible, particularly because of the strong antagonistic effect of pseudonocardiaceous secretions on the cultivated fungi.

Like any soil-dwelling insect, ants continually accumulate microbes on their integument, particularly in areas that are recessed and difficult to clean (e.g., the sternum between the legs). Most of these microbial accretions will have neutral or detrimental effects on an ant, but such unavoidable and predictable associations can serve as the raw material for the evolution of ant-microbe mutualisms. Under this view, only some but not all integumental microbes are beneficial, even if specific microbes occur at high abundance on the integument and are sustained inadvertently as a byproduct of cuticular secretion. A disease interpretation of all integumental actinomycetes is inconsistent with 2 findings, however. First, *Pseudonocardia* accumulates preferentially on apparently derived cuticular structures (14); and second, *Pseudonocardia* abundance on *Acromyrmex octospinosus* workers appears to increase when a nest is experimentally infected with *Escovopsis*, as if workers up-regulate *Pseudonocardia* abundance in response to *Escovopsis* infection (37). To rule out ant-actinomycete and cultivar-actinomycete antagonism for any particular attine lineage, it will be critical to establish whether the ants indeed evolved and maintained cuticular features to protect and nourish specific actinomycete associates (14) or whether the microbial associates are adventitious invaders that take advantage of inert cuticular accretions that the ants accumulate for other purposes.

If pseudonocardiaceous associates of attine workers function as mutualists, it appears that their primary role is not to supply antibiotics for the specific purpose of suppressing *Escovopsis*, as is widely believed (5, 18, 20, 21, 22, 24, 27, 30–33). Likewise, our antifungal assays (Table 2, Fig. S5) do not support the hypothesis that the pseudonocardiaceous bacteria specifically suppress entomopathogenic diseases of the ants, or endophytic and saprotrophic intruders in gardens. An integumental bacterial coat might protect the ants against bacterial or fungal infections to which the ants are exposed during their continuous shoulder rubbing with the microbial biofilms in their gardens. If so, the pseudonocardiaceous accretions on the integument may then complement or enhance the general antimicrobial role of metapleural gland secretions for protection of ants (41). This hypothesis could also explain why garden workers need and actually show higher *Pseudonocardia* loads than foragers (18, 37). Lastly, it is also possible that the ants infuse the walls of garden chambers with pseudonocardiaceous secretions to prevent uncontrolled spread of cultivar mycelium.

One severe criticism pertaining to the above mutualism hypotheses is that it remains unclear how the ants control the spread on their bodies of actinomycete variants that do not carry desirable antibiotic traits. Specifically, preventing the invasion of nonbeneficial actinomycete mutants arising from beneficial types, or preventing the invasion of nonbeneficial microbes invading from external sources, is likely a severe problem to the ants because it is actually not in the short-term evolutionary interests of the cuticular microbes to solve any disease problems of the ants or the cultivars. Instead, under microbe–microbe competition for the same nutrients on the ants, the cuticular microbes are selected in the short run to maximize their own growth rates, and the bacteria are therefore expected to jettison any metabolically costly production of antibiotics that attenuate their growth rate. Antibiotics secreted by cuticular microbes are therefore most likely maintained evolutionarily if they serve the interests of the microbes (i.e., by contributing to success in microbe–microbe competition for cuticular resources), and any antibiotic activities against garden diseases such as *Escovopsis* therefore could be coincidental byproducts. Consequently, the key parameters that need to be elucidated are not only the efficiencies of any vertical versus horizontal transmission of cuticular microbes, as emphasized in the prevailing ant-*Pseudonocardia* models (5, 18, 20, 22, 23), but more critically (*a*) the frequency at which nonbeneficial mutants arise from any beneficial types on the ant integument (even under strict vertical transmission), (*b*) the frequency at which nonbeneficial microbes colonize the ants from external sources, and (*c*) the effectiveness of any mechanisms that the ants may have (or not have) to eliminate such nonbeneficial bacterial associates.

A New Model of Ant-Cultivar-Actinomycete Association. The accumulated evidence prompts revision of the prevailing attine ant-*Escovopsis*-*Pseudonocardia* coevolution model along the following lines. (*i*) The roles of *Pseudonocardia* on the attine integument are likely to be diverse; not all may be mutualists. Future studies will need to document experimentally whether the presence or absence of bacterial associates indeed enhances the fitness of any ant host. (*ii*) *Pseudonocardia* and other integumental actinomycetes possess nonspecific antifungal properties. Because of the generalized antifungal activity, documentation of antibiosis against *Escovopsis* is insufficient to implicate a mutualistic role of *Pseudonocardia*. Moreover, *Pseudonocardia* secretions may inhibit *Escovopsis* not because of special antibiotic potency but because *Escovopsis* is readily inhibited, as *Escovopsis* is even suppressed by garden yeasts (8), a group of microbes not known to be rich in antibiotics. At present, there is no evidence that any attine-associated microbe is evolutionarily derived to specifically suppress *Escovopsis*. (*iii*) Multiple bacterial lineages with diverse antimicrobial properties grow consistently on attine ants, and there is no evidence that any of these consistent associates is vertically transmitted over many ant generations. Rather, consistent association with commensal, detrimental, or mutualistic *Pseudonocardia* (and other microbes) may occur because of predictable, de novo bacterial colonization of the ant integument from environmental sources (17, 36). Future studies should determine how many of these coexisting microbial lineages compete in situ (and thus could evolve competitive traits that harm the ants) and how many of them may complement each other's function as potential mutualists of the ants. (*iv*) Because pseudonocardiaceous secretions can severely harm the lepiotaceous cultivars, any application of secretion would have to be local [e.g., targeting critically diseased garden parts (41)] and the ants should prevent the spread of secretions across the garden at large. Rather than garden hygiene, possible alternate mutualistic roles of integumental microbes could include protection of the ants (Fig. S4*E*) or sanitation of the nest environment (suppression of microbes that colonize nest walls or degrade nest structures). Future studies should test for both nonadaptive and adaptive roles of integumental microbes in carefully designed experiments.

Materials and Methods

Ant Colonies. Actinomycete bacteria were isolated from 8 lab colonies of 6 attine species: *T. zeteki* ($n = 2$) and *S. amabilis* ($n = 1$) collected originally in Panama; *T. septentrionalis* ($n = 1$) from Louisiana; *T. turrifex* ($n = 1$) and *C. wheeleri* ($n = 1$) from Texas; and *M. smithii* ($n = 2$), one colony from Panama, one colony from Argentina (Table S3). The colonies had been kept in the laboratory at the University of Texas, Austin, TX, for 3–7 years before actinomycete isolation. Lab colonies experience higher *Escovopsis* pressure than field colonies, and it is difficult to prevent *Escovopsis* cross-infection of lab colonies (9, 37); the studied laboratory colonies therefore continued to be exposed to *Escovopsis* even after removal from the field, but exposure to other microbes likely altered the microbial-ecological conditions of the studied colonies. The sample included primarily ants from the genera *Trachymyrmex* and *Cyphomyrmex* because *Pseudonocardia* bacteria appear to occur abundantly on the integument of workers in these 2 genera (14, 17) and because *T. zeteki* was studied extensively before (9, 13, 14, 20).

Isolation of Actinomycete Test Species. Actinomycetes were isolated on chitin-medium described by (13) and (14). Our basic protocol replicated the isolation protocol of these previous studies, with only minor changes (see *SI Methods*). Individual workers were taken with sterile forceps from garden chambers of the laboratory ant colonies then vortexed for 10 min in 1 ml saline buffer (see *SI Methods*) to dislodge microbes from the ant integument. For the 4 *Trachymyrmex* colonies, one garden worker was vortexed per colony. Because of the small size of *C. wheeleri* and *M. smithii* workers, and because little integumental accretion was visible on ants, 10 garden workers were pooled per colony from these 2 species. For each ant colony, suspensions were spread on 2 chitin plates, one with 50 μl and one with 500 μl suspension. The 50 μl dilution allowed for more reliable bacterial isolation. For the *Trachymyrmex* colonies, we additionally scraped the accretion from the propleural plate of a single worker with a sterilized needle and streaked the accretion onto chitin medium, as described in (13). Chitin plates were kept at room temperature. The first actinomycete colonies were visible after 8–10 days. Colonies were picked from each plate 10 days and again 21 days after initial inoculation, then transferred to antibiotic-free yeast malt extract agar [YMEA; 0.4% yeast extract; 1% malt extract; 0.4% dextrose, 1.5% agar; (14)]. The growth of the ant-associated actinomycetes on antibiotic-free chitin plates appears faster than on the antibiotic-supplemented culture plates used in previous studies (13, 14, 20). We isolated all visible actinomycete morphotypes for subsequent antifungal challenges and identification via 16S sequencing.

Repeat Isolations of Actinomycetes. To confirm the consistency of the dominant actinomycete species [the resident species *sensu* (20)], we repeated the isolations again after 3 months. In the repeat isolation, we pooled 5–10 workers per nest for vortexing, spread the suspension at 50 μl/plate on 3 chitin plates, then subcultured all visible actinomycete morphotypes for identification with 16S sequencing. In these repeat isolations, we included *S. amabilis*, which was not screened in our initial survey.

Comparing Numbers of *Amycolatopsis* and *Pseudonocardia* in Plates. *Pseudonocardia* and *Amycolatopsis* bacterial colony forming units (CFU) were counted on the chitin-medium plates 2 weeks after spreading the bacterial suspensions, which were obtained by vortexing as described above. *Pseudonocardia* colonies were identified by their white button-like compact appearance; *Amycolatopsis* colonies were identified by their filamentous fuzzy appearance. For each plate of the repeat isolation, 8 random 1 cm $\times$ 1.3 cm patches were surveyed under the microscope, and numbers of *Pseudonocardia* and *Amycolatopsis* CFUs were counted in each patch then compared in a Wilcoxon sign-rank test.

Taxonomic Identification of Actinomycetes with 16S Sequencing. A small sample of actinomycete growth was lifted from a pure live culture (on YMEA medium) and extracted using a standard Chelex protocol (Sigma-Aldrich). Bacterial isolates were characterized by sequencing a segment of the 16S rDNA gene using the primer pairs U519F and 1406R (42) or AMP2 and AMP3 (43) (see *SI Methods*). All sequences were compared via the BLAST to information available at GenBank in March 2009.

Tag-Encoded FLX 454-Pyrosequencing (bTEFAP). Whole bacterial communities associated with ants and gardens were quantified with tag-encoded titanium amplicon pyrosequencing, as described previously (44) (see *SI Methods*). In short, raw sequences from bTEFAP were screened and trimmed based upon quality scores and binned into individual sample collections. Sequence collections were then depleted of short reads ($<$ 200 bp) and of chimeras using B2C2. The remaining sequences were assigned to bacterial species using BLASTn comparison with a high-quality 16S-database derived from National Center for Biotech-

nology Information and curated at the Medical Biofilm Research Institute. Tag-encoded 454-pyrosequencing yielded a total of 41,561 16S-sequences from 4 ant samples and 4 gardens (from *M. smithii*, 2 nests; *C. wheeleri*, and *T. septentrionalis*), with an average sequence length of 457 bp (see *SI Text*). Pyrosequencing reads are deposited at GenBank under accession SRA008625.9.

Isolation of Filamentous Test-Fungi from Attine Ants and Gardens. Sources of test-fungi and isolation procedures are detailed in Table S4 and *SI Methods*. In short, we isolated 7 cultivar fungi from 7 laboratory colonies; 6 endophytic and saprotrophic fungi from 4 laboratory colonies; 2 garden pathogens from 2 laboratory colonies and 2 more from glycerol-stored samples; 3 ant pathogens from 3 laboratory colonies; and 2 general entomopathogenic fungi.

Antibiotic Challenges. The antifungal effect of the 12 actinomycete isolates was quantified using a modified protocol of (13) and (14). An actinomycete isolate was inoculated in the center of a YMEA plate (8.5 cm diameter), then allowed to grow at room temperature for about 2 weeks (because of logistical constraints, the duration varied slightly between actinomycete species, but not between replicate plates within actinomycete isolates). This growth period of 2 weeks was shorter than the 3-week growth period used by previous researchers to assess the antibiotic properties of ant-associated actinomycetes (13, 20), and our assays therefore test at lower antibiotic concentrations than previous researchers. An agar plug of about $3 \times 3\ mm^2$ was then cut from the growth front of a test-fungus (subcultured onto a new PDA plate within 4 weeks before the experiment and grown on PDA without antibiotics), and the plug was then placed halfway between the growth front of the actinomycete and the edge of the Petri plate (Fig. S4). Each confrontation was replicated within the same test-plate by placing a second plug diametrically opposite to the first plug. The location of the plug was then traced on the reverse of the test-plate to mark the origin of mycelium growing from the plug laterally across the test-agar.

The growth of each test-fungus was measured for one month (once every 4 days). Using a caliper (0.05 mm accuracy) held against the reverse of a plate, 2 measures of mycelial growth were taken for each plug, one for growth *toward* the actinomycete culture, one for growth *away* (Fig. S4). The assay therefore measured relative growth of test-fungi in a gradient of actinomycete secretions emanating from the actinomycete culture in the center of the plate. To prevent any a priori growth bias of test-fungi toward or away from the actinomycete culture, each plug was oriented such that the sides with the newer and older mycelial growth in the plug did not face toward the center nor the outside of the plate. As a control, each test fungus was inoculated on a plate without any actinomycete. Some test-fungi sprouted aerial mycelium from the plug, but did not grow laterally across or into the medium. Growth of such fungi was scored as zero, as the assay aimed at assessing growth of mycelium that interacted with the gradient of actinomycete secretions.

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Supporting Information

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SI Methods

Isolation of Actinomycete Test Species. Actinomycetes were isolated on chitin-medium described by (1) and (2). Our basic protocol replicated the isolation protocol of these previous studies, with 2 exceptions. The 2 exceptions in our study were: (*a*) use of a saline buffer (rather than distilled water) to suspend bacteria when dislodging microbes from the ants through vortexing and (*b*) use of antibiotic-free medium for actinomycete isolation. Previous studies had supplemented isolation plates with antibiotics (nystatin and cycloheximide) to suppress growth of fungi during bacterial isolation, but we used antibiotic-free medium to permit simultaneous isolation of integument-inhabiting fungi (e.g., entomopathogens) that could be useful for testing of the antifungal properties of attine actinomycetes. Because all microbes grow very slowly on the minimum-carbon chitin medium, and because actinomycetes grew abundantly on culture plates, actinomycetes could be readily isolated from the chitin plates on the first attempt.

Buffer for Suspension of Bacteria During Vortexing. The saline buffer contained the same salt concentrations as the chitin-medium (0.7g K_2HPO_4, 0.5g $MgSO_4$, 0.3g KH_2PO_4, 0.01g $FeSO_4$, 0.001g $ZnSO_4$ dissolved in 1 liter ultrapure water).

PCR Conditions and Sequencing. Bacterial isolates were characterized by sequencing a segment of the 16S rDNA gene using the primer pair U519F and 1406R (3) (1 μl 10x buffer, 0.8 μl $MgCl_2$ 25 mM, 0.8 μl dNTP mix [2.5 mM each nucleotide], 0.8 μl 100x BSA, 0.6 μl of each primer at 2 mM, 0.1 μl Taq polymerase, 1 μl template, and ddH20 to a total volume of 10 μl). The PCR temperature profile was 94 °C for 1 min, 50 °C for 1 min, 72 °C for 2 min; repeat for 35 cycles; followed by a final extension step of 72 °C for 10 min. All PCR products were cycle-sequenced with the ABI Big Dye Terminator Kit (version 3.1) on an ABI PRISM 3100 automated sequencer.

Bacterial Tag-Encoded Titanium Amplicon Pyrosequencing. DNA was extracted from ants and gardens after dry ice methanol freezing and mortar and pestle grinding to a fine powder using methods detailed previously (4). Homogenized powder was resuspended in 500 μl RLT buffer (QIAGEN) (with β- mercaptoethanol). A sterile 5 mm steel bead (QIAGEN) and 500 μl 0.1 mm glass beads (Scientific Industries, Inc.) were added for complete bacterial lyses in a Qiagen TissueLyser (QIAGEN), run at 30 Hz for 5 min. Samples were centrifuged briefly, and 100 μl 100% ethanol were added to a 100 μl aliquot of the sample supernatant. This mixture was added to a DNA spin column, and DNA recovery protocols were followed as instructed in the QIAamp DNA Mini Kit (QIAGEN) starting at step 5 of the Tissue Protocol. DNA was eluted from the column with 30 μl water and samples were diluted accordingly to a final concentration of 20 ng/μl for use with SYBR Green RT-PCR (Qiagen). DNA samples were quantified using a Nanodrop spectrophotometer (Nyxor Biotech). Bacterial tag-encoded titanium amplicon pyrosequencing and data processing were performed as described previously (5, 6). In short, raw data from bTEFAP was screened and trimmed based upon quality scores and binned into individual sample collections. Sequence collections were then depleted of chimeras using B2C2. The resulting files were then depleted of short reads (<200 bp) and bacterial species identified using BLASTn comparison to a curated high quality 16S database derived from National Center for Biotechnology Information (NCBI). Data were compiled and relative percentages of a given bacterial species were determined for each sample. Data were also compiled at each individual taxonomic level according to the NCBI taxonomy criteria as described previously (5, 6). Collection and sequence information is deposited at GenBank under accessions SRA008625.9.

Isolation of Ecologically-Relevant Test Fungi. To accumulate a set of fungi (Table S4) for the testing of antifungal activities of the actinomycete isolates, attine cultivars and "weed" microfungi were isolated from attine gardens of the same nests from which actinomycetes had been obtained. Fungi were isolated from gardens about 6 weeks after the first actinomycete isolation (see *SI Methods S5* for isolation procedure). Isolations were performed by carefully placing 8 garden fragments (2–4 mm diameter) with sterilized forceps on potato dextrose agar (PDA), using the methods of (7) for isolations of cultivars and using the methods of (8) for the isolation of noncultivar garden microfungi. Cultivars were obtained from all of the 7 nests, but noncultivar microfungi were obtained only from 5 of the 7 nests (Table S4). All microfungi obtained from attine gardens were used for testing except for 2 *Penicillium* isolates, which were excluded because of the great risk of contaminating the work environment with spores. One *Escovopsis* strain was isolated from the experimental nest of *C. wheeleri*. Two additional *Escovopsis* strains (one from *T. zeteki* from Panama, one from *T. turrifex* from Texas) that had been obtained in previous *Escovopsis* surveys were added to the set of test-fungi. These 2 strains had been stored under glycerol at −80 °C since isolation in 2003 and 2006, respectively, but were revived 3 weeks before testing in 2008. Three filamentous fungi obtained from the chitin plates (see above; vortex of whole workers of *M. smithii*, *T. turrifex*, and *C. wheeleri*) were also added to the set of test-fungi. One facultatively entomopathogenic fungus, *Fusarium solani*, was isolated from an *Atta texana* queen that had died in an incipient lab nest during spring 2008. One additional entomopathogenic fungus (*Acrodontium* sp.) was isolated from a diseased queen of *Acromyrmex versicolor* that had been collected from a mating flight in Arizona in 2007, then reared in a lab nest. Apart for the exclusion of the 2 *Penicillium* isolates mentioned above, the complete set of 14 noncultivar test fungi (Table S4) represents an unbiased selection of filamentous fungi available in the Mueller Lab shortly after isolation of the actinomycete species in 2008. Because these filamentous fungi were all isolated from attine gardens or from attine ants, the set of 14 test-fungi should be more representative for the problem fungi that ants encounter than standard laboratory species used traditionally for antibiotic testing. To increase the number of entomopathogenic test fungi, we added 5 *Beauveria* and one *Metarhizium* strains from the collection of Department of Agriculture–Agriculture Research Service (USDA-ARS) Plant Protection Research Unit, U.S. Plant, Soil and Nutrition Laboratory, Ithaca, NY 14853-2901.

Test-fungi were identified by sequencing of the ITS rDNA region using the universal primers ITS4 x ITS5 (9) and the LSU rDNA region using the universal primers LR5 x LROR (10). Sequences were compared via the BLASTn with information available at GenBank in September 2008. BLASTn results are listed in Table S4.

SI Results

Presence of Diverse Pseudonocardiaceous Bacteria in Single Attine Nests. Tag-encoded 454-pyrosequencing yielded a total of 41,561 16S-sequences from 4 ant samples and 4 gardens (from *M.*

smithii, 2 nests; *C. wheeleri*, and *T. septentrionalis*) with an average sequence length of 457 bp. The garden sample of *T. septentrionalis* had to be discarded because it consistently yielded inadequate reads (fewer than 30 reads) in repeat 454-sequencing attempts. For the remaining samples, an average of about 5,400 16S-amplicons were characterized for worker-associated bacterial communities and an average of 6,600 amplicons for garden-associated bacterial communities. Rarefaction analyses indicate that this sampling regime of about 5,000 16S-amplicons per community appears sufficient to capture a significant proportion (if not most) of the bacterial diversities (Table S1, Figs. S1 and S2). Under the most stringent definition of Operational Taxonomic Units (OTUs; at least 1% sequence difference between OTUs), accumulation curves reveal 200–300 OTUs (observed) and 300–600 OTUs (predicted with additional sampling) for worker-associated bacterial communities, and 200–500 OTUs (observed) and 200-1000 OTUs (predicted) for garden-associated communities (Fig. S1, Table S1). Compared to other known bacterial communities (e.g., ref. 11), attine-associated bacterial communities emerge as moderately species-rich, even when using a stringent definition of OTUs (1% sequence difference). Additional diversity and richness indices [Shannon diversity, Chao1 richness, ACE richness; Table S2] indicate that bacterial communities in the 2 gardens of *M. smithii* were moderately richer and more diverse (by about a factor of 2) than the corresponding communities on worker ants, whereas ant-associated bacterial communities were richer and more diverse than garden-associated communities in *C. wheeleri*. Because of the small number of samples screened, these diversity patterns should not be over-interpreted.

***Amycolatopsis* Identification in the Present Versus Previous Studies.** Previous culture-dependent studies had failed to identify *Amycolatopis* from attine workers, for several reasons: First, *Amycolatopsis* was present only in *Mycocepurus smithii* samples, a species which had been screened only in one previous study (12). Second, because *Amycolatopsis* colonies exhibit mycelia-like fuzzy growth on the minimum-carbon isolation medium (unlike the button-like growth morphology typical for *Pseudonocardia*), previous researchers may have failed to recognize this growth form as an actinomycete. Third, fungicidal supplements in previous isolations could have precluded *Amycolatopsis* isolation on fungicidal medium but allowed such isolation on our antibiotic-free medium.

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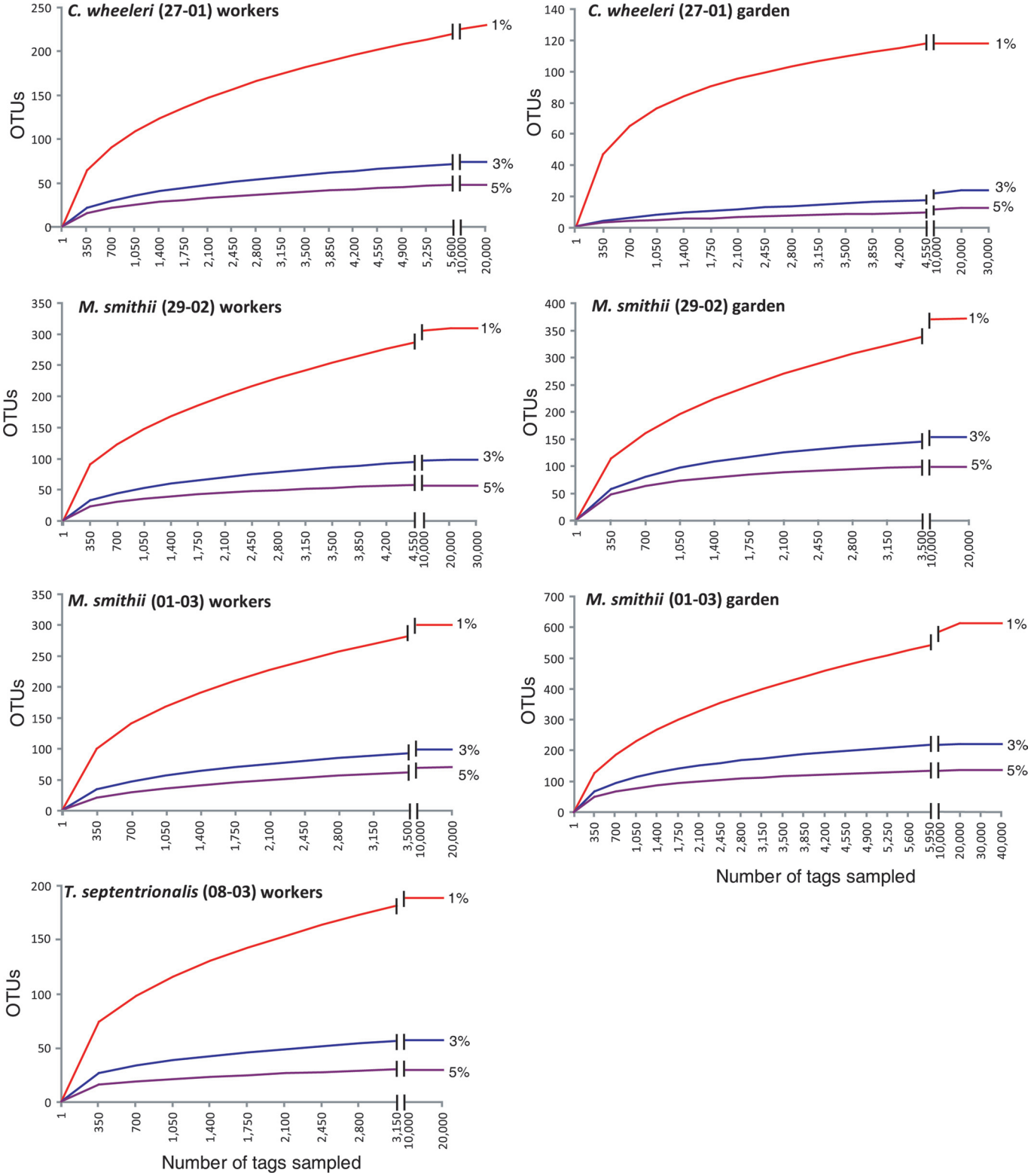

Fig. S1. Operational taxonomic unit (OTU)-accumulation curves and rarefaction predictions of OTU-diversity for each of the 7 bacterial communities screened. For each community, accumulation curves and rarefaction predictions are shown for 3 different stringency levels of defining OTUs (at least 1%, 3%, or 5% sequence difference between OTUs).

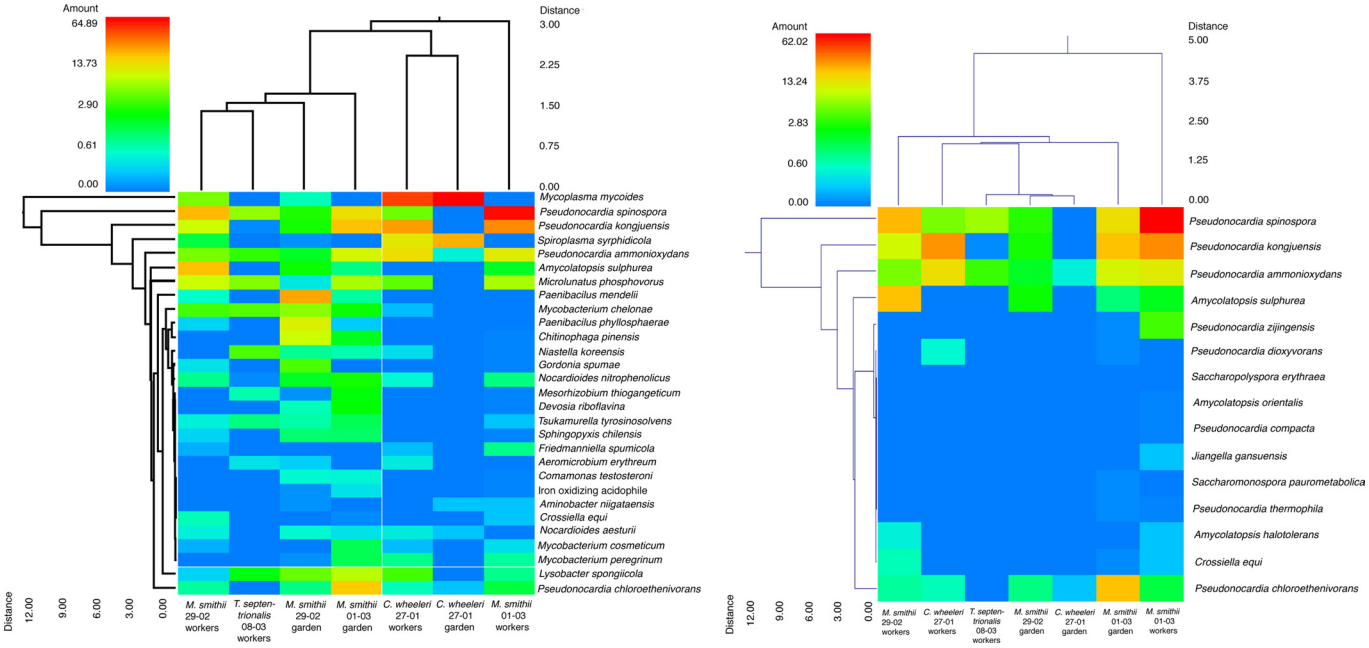

Fig. S2. Bacterial species identification and community composition obtained from 16S 454 pyrosequencing of worker-associated and garden-associated bacteria species (*Left*) and pseudonocardiaceous species (*Right*) in 4 attine ant colonies. Bacteria from the same source are depicted in individual columns. The dendrograms do not depict phylogenetic relationships; rather, dendrograms cluster bacteria and communities by relative bacterial abundances. Contribution percentage of each bacteria is given in Table S1.

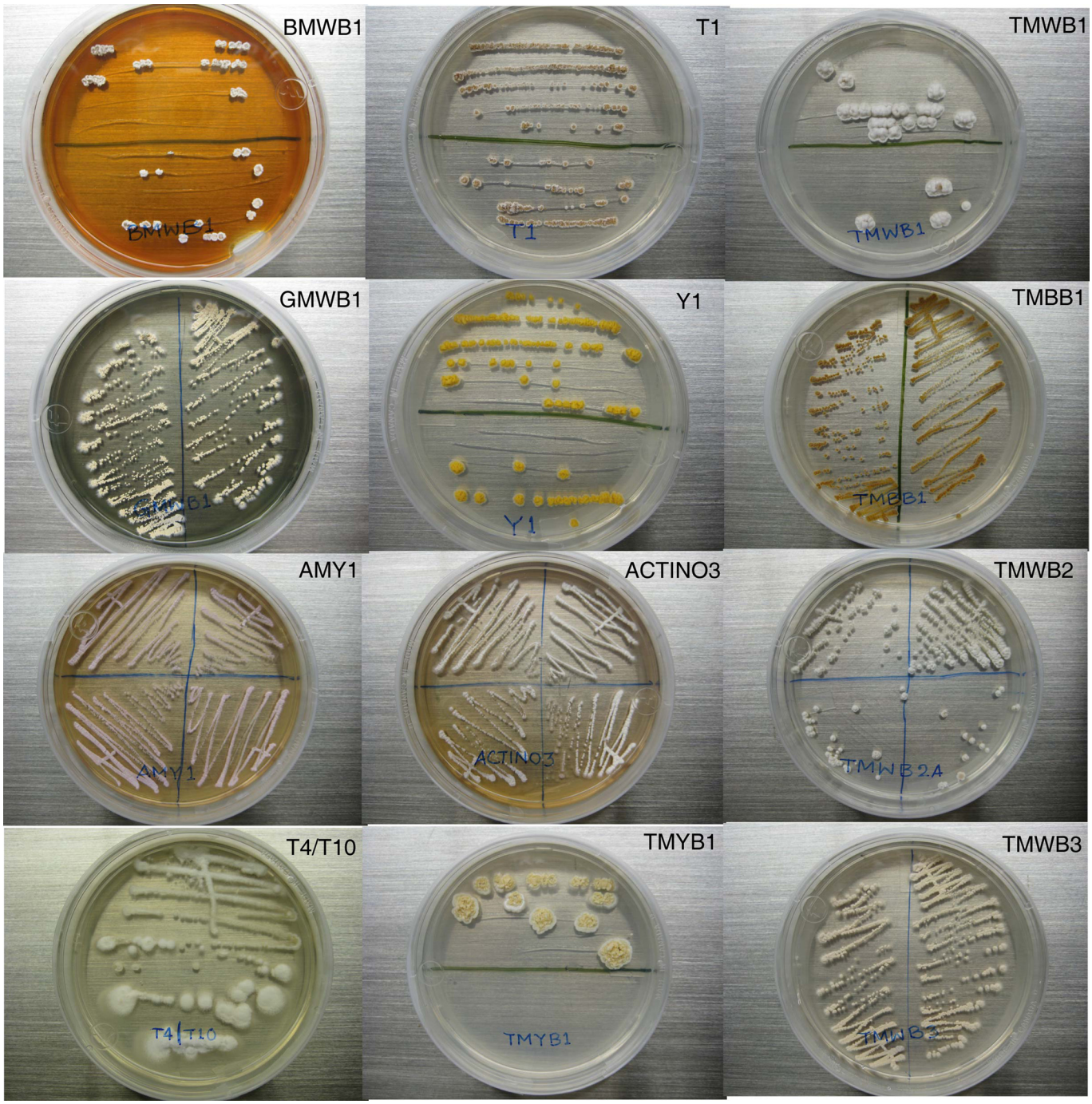

Fig. S3. Bacterial morphotypes growing on PDA agar at room temperature (see Table 2 for sources of isolation).

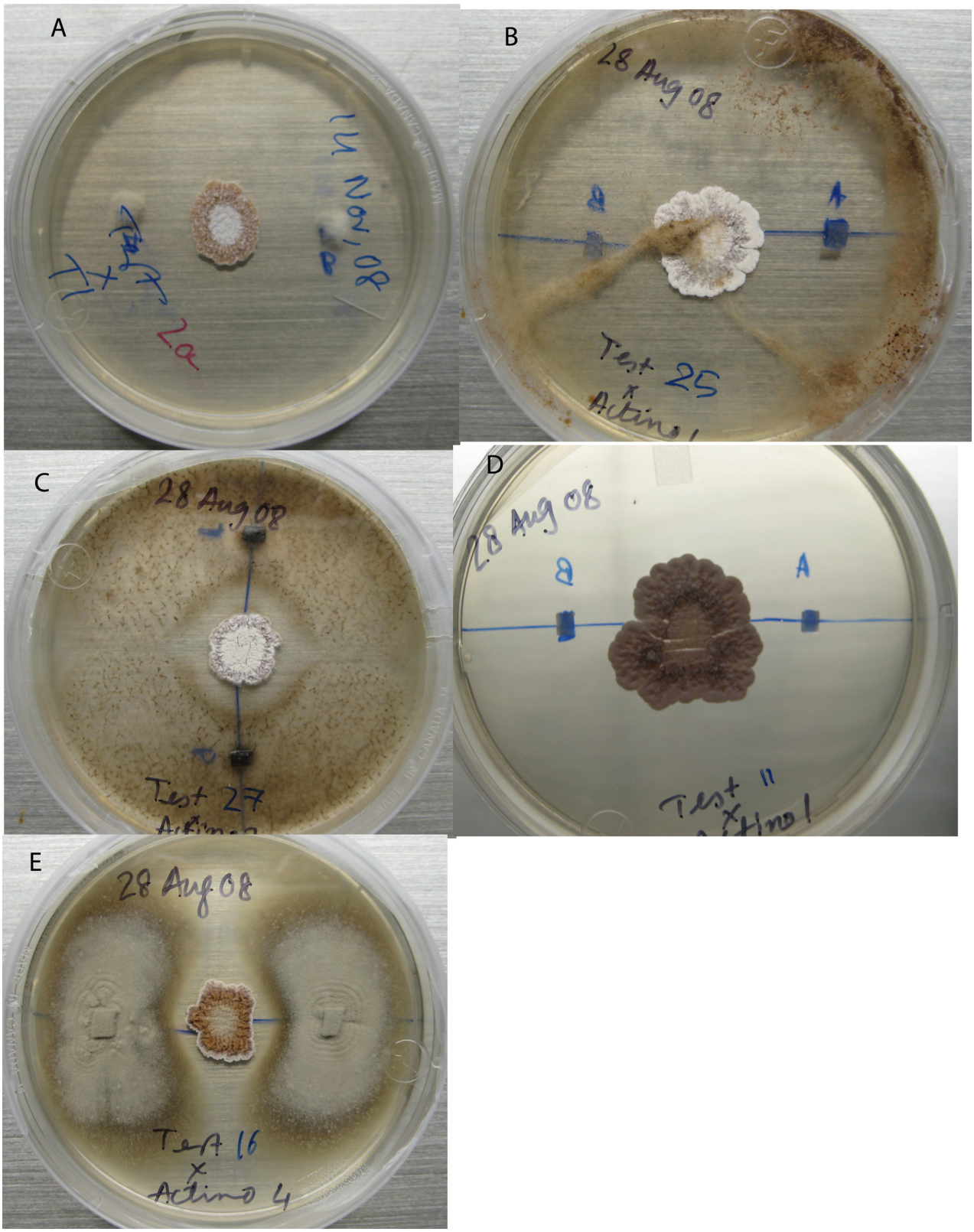

Fig. S4. (*A–E*). Assays testing antibiotic activity of *Pseudonocardia* (center of plate) against the same test fungus inoculated at 2 sides on the same plate. All photos were taken 28 days after inoculating the respective test fungus. (*A*) *Pseudonocardia*T1 vs. *Escovopsis*; (*B*) *Pseudonocardia*1 vs. *Escovopsis*; (*C*) *Pseudonocardia*2 vs. *Phoma*; (*D*) *Pseudonocardia*1 vs. cultivar; (*E*) *Pseudonocardia vs. entomopathogen*.

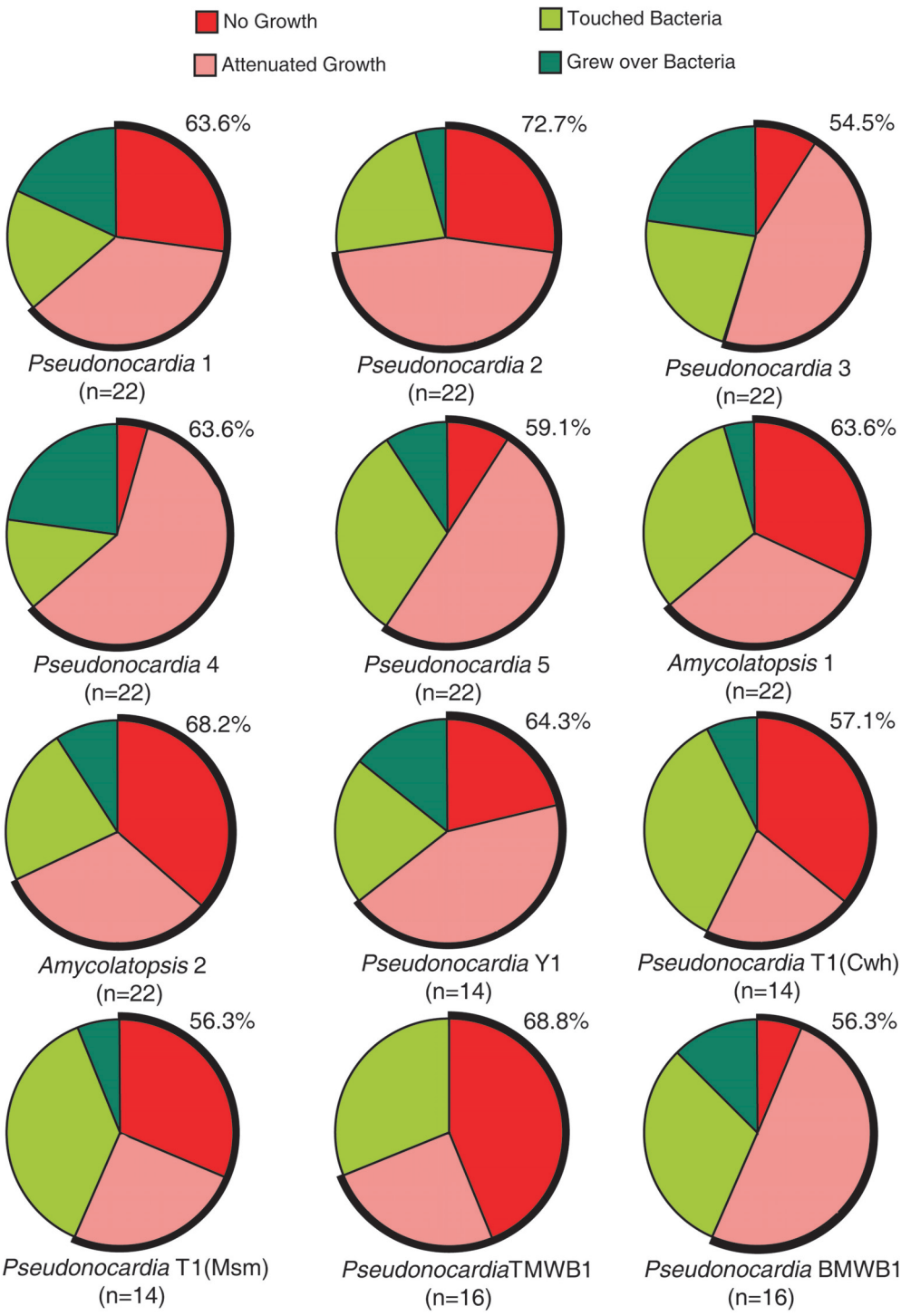

Fig. S5. Inhibition profile of *Pseudonocardia* and *Amycolatopsis* confronted with attine cultivars or different types of problem fungi. The extent of inhibition was scored as complete inhibition (no growth of test fungi), attenuated growth compared to control growth, and 2 types of no inhibition (fungi grew up to and touched the test bacterium but did not grow over the bacterium; test fungi grew over the test bacterium). The pie charts represent percentages of different growth responses by test fungi when challenged with a particular pseudonocardiaceous bacterium. Red sectors represent inhibited growth (total percentage given on the right side of each pie chart), green sectors unaffected growth; n is the number of fungi tested against each bacterium. (see Tables S3 and S4 for sources of isolation of bacteria and fungi, respectively). Authors will be able to provide raw data for each challenge upon request.

Table S1. Percent contribution of bacterial species to attine bacterial communities, surveyed by 454 16S-amplicon pyrosequencing

Species	*Myco. smithii* 29–02 Worker	*Myco. smithii* 29–02 Garden	*Myco. smithii* 01–03 Worker	*Myco. smithii* 01–03 Garden	*Cypho. wheeleri* 27–01 Worker	*Cypho. wheeleri* 27–01 Garden	*Trachy. septentrionalis* 08–03 Worker
Achromobacter cf. xylosoxidans	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Acidovorax cf. avenae	0.000	0.278	0.000	0.000	0.000	0.000	0.000
Acinetobacter cf. calcoaceticus	0.055	0.000	0.000	0.000	0.000	0.000	0.000
Acinetobacter cf. junii	3.324	0.000	0.000	0.000	0.000	0.000	0.000
Actinotalea cf. fermentans	0.027	0.040	0.000	0.254	0.000	0.000	0.036
Aeromicrobium cf. alkaliterrae	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Aeromicrobium cf. erythreum	0.027	0.079	0.000	0.000	0.128	0.000	0.109
Aeromicrobium cf. marinum	0.027	0.000	0.000	1.050	0.000	0.000	0.615
Afipia cf. felis	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Afipia cf. massiliensis	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Agrococcus cf. jenensis	0.000	0.000	0.000	0.000	0.096	0.000	0.471
Agromyces cf. italicus	0.027	1.626	0.000	0.000	0.000	0.000	0.000
Agromyces cf. ramosus	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Agromyces cf. ulmi	0.027	1.705	0.000	0.000	0.000	0.000	0.000
Alcaligenes cf. faecalis	0.000	0.357	0.000	0.000	0.000	0.000	0.217
Alistipes cf. putredinis	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Amaricoccus cf. kaplicensis	0.000	0.000	0.033	0.797	0.000	0.000	0.000
Amaricoccus cf. macauensis	0.027	0.040	0.000	20.029	0.000	0.000	0.000
Aminobacter cf. niigataensis	0.000	0.040	0.065	0.000	0.000	0.066	0.000
Amycolatopsis cf. halotolerans	0.137	0.000	0.065	0.000	0.000	0.000	0.000
Amycolatopsis cf. orientalis	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Amycolatopsis cf. sulphurea	12.802	0.991	0.717	0.326	0.000	0.000	0.000
Aurantimonas cf. coralicida	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Azospirillum cf. brasilense	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Bacillus cf. caldolyticus	0.000	0.159	0.000	0.362	0.000	0.000	0.000
Bacillus cf. mannanilyticus	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Bacteroides cf. splanchnicus	0.000	0.000	0.065	0.036	0.000	0.133	0.109
Bacteroides cf. vulgatus	0.000	0.000	0.000	0.000	0.000	0.000	0.000
Blastochloris cf. sulfoviridis	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Bordetella cf. hinzii	0.027	0.317	0.000	0.000	0.000	0.000	0.000
Bosea cf. minatitlanensis	0.000	0.040	0.000	0.109	0.000	0.000	0.109
Bosea cf. vestrisii	0.027	0.040	0.000	0.000	0.000	0.000	0.000
Brachybacterium cf. nesterenkovii	0.000	0.000	0.033	0.217	0.000	0.000	0.000
Brachybacterium cf. sacelli	0.000	0.000	0.033	0.616	0.000	0.000	0.000
Bradyrhizobium cf. group	0.000	0.040	0.000	0.435	0.000	0.000	0.000
Bradyrhizobium cf. japonicum	0.000	0.000	0.000	1.014	0.000	0.000	0.000
Bradyrhizobium cf. liaoningense	0.000	0.198	0.000	0.000	0.000	0.000	0.000
Brevibacillus cf. borstelensis	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Brevibacillus cf. formosus	0.000	0.000	0.000	0.109	0.000	0.000	0.000
Brevibacillus cf. levickii	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Brooklawnia cf. cerclae	0.000	0.000	0.000	0.000	0.000	0.000	0.036
Burkholderia cf. ambifaria	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Burkholderia cf. cenocepacia	0.000	0.278	0.000	0.072	0.000	0.000	0.072
Burkholderia cf. cepacia	0.000	0.079	0.000	0.072	0.000	0.000	0.000
Burkholderia cf. pyrrocinia	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Burkholderia cf. thailandensis	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Candidatus cf. Nostocoida	0.000	0.000	0.000	0.036	0.000	0.000	0.109
Candidatus cf. Protochlamydia	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Candidatus cf. Reyranella	0.000	0.000	0.000	0.109	0.000	0.000	0.000
Candidatus cf. Rhizobium	0.000	0.238	0.000	0.000	0.000	0.000	0.000
Candidatus cf. Xiphinematobacter	0.000	0.159	0.000	0.000	0.000	0.000	0.000
Cellulomonas cf. denverensis	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Cellulosimicrobium cf. funkei	0.000	0.198	0.000	0.942	0.000	0.000	0.000
CFB cf. group	0.027	0.000	0.033	0.217	0.000	0.000	0.000
Chitinophaga cf. pinensis	0.027	5.115	0.033	0.724	0.000	0.000	0.000
Chryseobacterium cf. joostei	0.604	0.000	0.000	0.000	0.000	0.000	52.009
Clostridium cf. propionicum	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Comamonas cf. testosteroni	0.000	0.159	0.033	0.145	0.000	0.000	0.000
Conexibacter cf. woesei	0.055	0.278	0.000	0.217	0.000	0.000	0.651

Species	*Myco. smithii* 29–02 Worker	*Myco. smithii* 29–02 Garden	*Myco. smithii* 01–03 Worker	*Myco. smithii* 01–03 Garden	*Cypho. wheeleri* 27–01 Worker	*Cypho. wheeleri* 27–01 Garden	*Trachy. septentrionalis* 08–03 Worker
Crassostrea cf. virginica	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Crossiella cf. equi	0.192	0.000	0.065	0.036	0.000	0.000	0.000
Cupriavidus cf. basilensis	0.000	0.000	0.000	0.254	0.000	0.000	0.000
Cupriavidus cf. necator	0.000	0.000	0.000	0.616	0.000	0.000	0.000
Demetria cf. terragena	0.000	0.000	0.000	0.000	0.000	0.000	2.063
Dermabacter cf. hominis	0.000	0.000	0.000	0.109	0.000	0.000	0.000
Dermatophilus cf. congolensis	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Desulfovibrio cf. piger	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Devosia cf. limi	0.000	0.000	0.033	0.435	0.000	0.000	0.000
Devosia cf. riboflavina	0.027	0.198	0.033	0.942	0.000	0.000	0.000
Dokdonella cf. fugitiva	0.000	3.370	0.000	0.398	0.000	0.000	0.000
Dokdonella cf. koreensis	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Dyadobacter cf. fermentans	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Enhygromyxa cf. salina	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Ensifer cf. adhaerens	0.000	0.159	0.000	0.000	0.000	0.000	0.000
Entomoplasma cf. freundtii	0.000	0.000	0.000	0.000	0.000	0.000	0.109
Eubacterium cf. desmolans	0.000	0.000	0.000	0.000	0.032	0.000	0.000
Eubacterium cf. eligens	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Exiguobacterium cf. aestuarii	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Exiguobacterium cf. aurantiacum	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Faecalibacterium cf. prausnitzii	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Finegoldia cf. magna	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Flavobacterium cf. weaverense	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Friedmanniella cf. spumicola	0.055	0.000	0.293	0.000	0.064	0.000	0.000
Gordonia cf. namibiensis	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Gordonia cf. polyisoprenivorans	1.264	9.080	0.033	0.000	0.000	0.000	0.072
Gordonia cf. sinesedis	0.000	0.357	0.000	0.000	0.000	0.000	0.000
Gordonia cf. spumae	0.110	1.745	0.033	0.000	0.000	0.000	0.000
Gordonia cf. terrae	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Haemophilus cf. parainfluenzae	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Haliangium cf. tepidum	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Hespellia cf. porcina	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Hydrocarboniphaga cf. effusa	0.000	0.000	0.033	0.000	0.032	0.000	0.000
Hydrocoleum cf. lyngbyaceum	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Hydrogenophaga cf. intermedia	0.000	1.229	0.000	0.000	0.000	0.000	0.000
Hyphomicrobium cf. hollandicum	0.000	0.000	0.000	0.145	0.000	0.000	0.000
Hyphomicrobium cf. zavarzinii	0.000	0.040	0.000	0.109	0.032	0.000	0.000
iron-oxidizing cf. acidophile	0.000	0.040	0.033	0.109	0.000	0.000	0.000
Jiangella cf. gansuensis	0.000	0.000	0.065	0.000	0.000	0.000	0.000
Kaistia cf. adipata	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Kartchner cf. Caverns	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Klebsiella cf. pneumoniae	0.000	0.793	0.000	1.050	0.000	0.000	0.000
Kribbella cf. antibiotica	0.000	0.000	0.000	0.000	0.225	0.000	0.000
Kribbella cf. swartbergensis	0.000	0.000	0.098	0.507	0.000	0.000	0.000
Lactobacillus cf. acidophilus	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Legionella-like cf. amoebal	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Leifsonia cf. xyli	0.000	0.119	0.000	0.000	0.000	0.000	0.036
Leptospira cf. meyeri	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Lysobacter cf. spongiicola	0.082	2.220	0.293	4.346	1.670	0.000	0.796
Marmoricola cf. aurantiacus	0.000	0.000	0.000	0.000	0.000	0.000	0.072
Mesoplasma cf. chauliocola	0.000	0.000	0.000	0.000	0.514	1.064	0.000
Mesoplasma cf. lactucae	0.000	0.000	0.000	0.000	0.000	0.000	22.222
Mesoplasma cf. tabanidae	0.000	0.000	0.000	0.000	15.382	17.354	0.000
Mesorhizobium cf. amorphae	0.000	0.079	0.000	0.109	0.032	0.000	0.036
Mesorhizobium cf. chacoense	0.000	0.079	0.065	0.290	0.000	0.000	0.000
Mesorhizobium cf. loti	0.000	0.079	0.000	0.580	0.000	0.000	0.000
Mesorhizobium cf. plurifarium	0.000	0.040	0.000	1.340	0.000	0.000	0.000
Mesorhizobium cf. temperatum	0.000	0.040	0.000	0.290	0.000	0.000	0.000
Mesorhizobium cf. thiogangeticum	0.000	0.040	0.033	0.869	0.000	0.000	0.217

Species	*Myco. smithii* 29–02 Worker	*Myco. smithii* 29–02 Garden	*Myco. smithii* 01–03 Worker	*Myco. smithii* 01–03 Garden	*Cypho. wheeleri* 27–01 Worker	*Cypho. wheeleri* 27–01 Garden	*Trachy. septentrionalis* 08–03 Worker
Mesorhizobium cf. tianshanense	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Methylobacterium cf. specialis	0.000	0.000	0.000	0.000	0.032	0.133	0.036
Microbacterium cf. aurantiacum	0.000	0.000	0.033	0.326	0.000	0.000	0.000
Microbacterium cf. aurum	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. chocolatum	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. esteraromaticum	0.000	0.000	0.000	0.000	0.000	0.000	0.145
Microbacterium cf. flavescens	0.000	0.278	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. foliorum	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. hominis	0.000	0.000	0.000	0.109	0.000	0.000	0.398
Microbacterium cf. keratanolyticum	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. koreense	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. resistens	0.027	0.278	0.000	0.290	0.000	0.000	0.000
Microbacterium cf. thalassium	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Microbacterium cf. xylanilyticum	0.000	0.000	0.000	0.471	0.000	0.000	0.072
Microlunatus cf. phosphovorus	5.110	0.119	3.876	3.875	2.119	0.000	2.787
Mycobacterium cf. brisbanense	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Mycobacterium cf. chelonae	1.593	3.053	0.000	1.014	0.064	0.000	1.846
Mycobacterium cf. chlorophenolicum	0.000	0.000	0.033	0.036	0.000	0.000	0.000
Mycobacterium cf. conceptionense	0.000	0.000	0.000	0.688	0.000	0.000	0.000
Mycobacterium cf. conspicuum	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Mycobacterium cf. cosmeticum	0.055	0.000	0.098	0.471	0.064	0.000	0.000
Mycobacterium cf. elephantis	0.000	0.000	0.163	0.217	0.000	0.000	0.000
Mycobacterium cf. mageritense	0.000	0.000	0.033	0.217	0.000	0.000	0.000
Mycobacterium cf. parmense	0.000	0.000	0.033	0.036	0.000	0.000	0.000
Mycobacterium cf. peregrinum	0.027	0.040	0.261	0.471	0.289	0.000	0.000
Mycobacterium cf. phocaicum	0.000	1.190	0.098	0.036	0.000	0.000	0.217
Mycobacterium cf. poriferae	0.000	0.000	0.000	0.326	0.000	0.000	0.000
Mycobacterium cf. psychrotolerans	0.000	0.000	0.065	0.000	0.000	0.000	0.000
Mycobacterium cf. smegmatis	0.027	0.119	0.000	0.109	0.000	0.000	0.000
Mycoplasma cf. cottewii	0.110	0.000	0.000	0.000	0.032	0.000	0.000
Mycoplasma cf. mycoides	2.582	0.198	0.000	0.000	39.338	64.894	0.000
Nakamurella cf. multipartita	0.000	0.000	0.000	0.435	0.193	0.000	0.000
Neisseria cf. animalis	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Niastella cf. koreensis	0.027	0.278	0.033	0.217	0.096	0.000	1.701
Nitratireductor cf. aquibiodomus	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Nitrobacter cf. winogradskyi	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Nitrosococcus cf. oceani	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Nocardia cf. harenosa	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Nocardia cf. neocaledoniensis	0.000	0.000	0.065	0.072	0.000	0.000	0.000
Nocardia cf. paucivorans	0.000	0.000	0.065	0.000	0.000	0.000	0.000
Nocardioides cf. aestuarii	0.137	0.159	0.000	0.109	0.128	0.066	0.000
Nocardioides cf. alkalitolerans	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Nocardioides cf. aquiterrae	0.000	0.079	0.000	0.036	0.096	0.000	0.253
Nocardioides cf. dubius	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Nocardioides cf. ganghwensis	0.000	0.198	0.000	0.000	0.064	0.000	0.072
Nocardioides cf. kribbensis	0.000	0.040	0.000	0.072	0.000	0.000	0.000
Nocardioides cf. nitrophenolicus	0.302	0.714	0.326	1.123	0.161	0.000	0.036
Novosphingobium cf. pentaromativorans	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Ochrobactrum cf. anthropi	0.000	0.198	0.000	0.000	0.000	0.000	0.036
Ornithinicoccus cf. hortensis	0.000	0.000	0.000	0.000	0.000	0.000	0.109
Paenibacillus cf. chitinolyticus	0.000	0.000	0.000	0.254	0.000	0.000	0.000
Paenibacillus cf. mendelii	0.165	16.336	0.000	0.254	0.000	0.000	0.000
Paenibacillus cf. phyllosphaerae	0.082	6.661	0.000	0.072	0.000	0.000	0.000

Species	*Myco. smithii* 29–02 Worker	*Myco. smithii* 29–02 Garden	*Myco. smithii* 01–03 Worker	*Myco. smithii* 01–03 Garden	*Cypho. wheeleri* 27–01 Worker	*Cypho. wheeleri* 27–01 Garden	*Trachy. septentrionalis* 08–03 Worker
Papillibacter cf. cinnamivorans	0.000	0.000	0.000	0.036	0.000	0.000	0.036
Parachlamydia cf. acanthamoebae	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Paracoccus cf. denitrificans	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Paracoccus cf. pantotrophus	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Pelomonas cf. saccharophila	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Peptoniphilus cf. harei	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Peptoniphilus cf. ivorii	0.000	0.198	0.000	0.145	0.000	0.000	0.000
Phenylobacterium cf. falsum	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Phenylobacterium cf. koreense	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Phyllobacterium cf. bourgognense	0.000	0.634	0.000	0.000	0.000	0.000	0.000
Pigmentiphaga cf. kullae	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Planococcus cf. antarcticus	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Prevotella cf. oulorum	0.000	0.040	0.033	0.000	0.000	0.000	0.000
Prevotella cf. veroralis	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Promicromonospora cf. aerolata	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Propionibacterium cf. acnes	0.000	0.515	0.000	0.181	0.000	0.000	0.036
Propionicicella cf. superfundia	0.000	0.000	0.000	0.000	0.706	0.000	1.773
Propioniferax cf. innocua	0.000	0.000	0.000	0.000	0.128	0.000	0.000
Pseudaminobacter cf. salicylatoxidans	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Pseudomonas cf. aeruginosa	40.440	3.569	0.000	0.000	0.000	0.000	0.000
Pseudomonas cf. alcaligenes	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Pseudomonas cf. alcaliphila	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Pseudomonas cf. gessardii	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Pseudomonas cf. hibiscicola	1.429	0.198	0.000	0.000	0.000	0.000	0.000
Pseudomonas cf. mendocina	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Pseudomonas cf. otitidis	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Pseudonocardia cf. ammonioxydans	2.500	0.674	6.938	5.976	8.863	0.133	1.412
Pseudonocardia cf. chloroethenivorans	0.247	0.317	0.554	12.351	0.193	0.066	0.000
Pseudonocardia cf. compacta	0.000	0.000	0.033	0.000	0.000	0.000	0.000
Pseudonocardia cf. dioxanivorans	0.000	0.000	0.000	0.036	0.161	0.000	0.000
Pseudonocardia cf. kongjuensis	5.275	1.071	20.782	12.966	18.369	0.000	0.036
Pseudonocardia cf. spinosispora	13.874	1.229	62.020	8.982	2.473	0.000	3.330
Pseudonocardia cf. thermophila	0.000	0.000	0.033	0.036	0.000	0.000	0.000
Pseudonocardia cf. zijingensis	0.000	0.000	1.564	0.036	0.000	0.000	0.000
Pseudoxanthomonas cf. mexicana	0.000	9.794	0.033	0.000	0.000	0.000	0.000
Pseudoxanthomonas cf. spadix	0.000	10.151	0.000	0.000	0.000	0.000	0.000
Pseudoxanthomonas cf. suwonensis	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Ralstonia cf. insidiosa	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Ralstonia cf. mannitolilytica	0.000	0.000	0.000	0.000	0.000	0.000	0.760
Ralstonia cf. pickettii	0.000	0.159	0.000	0.000	0.000	0.000	0.000
Ralstonia cf. syzygii	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Rhizobium cf. gallicum	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Rhizobium cf. huautlense	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Rhizobium cf. loessense	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Rhodanobacter cf. spathiphylli	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Rhodobium cf. orientis	0.000	0.198	0.000	0.580	0.000	0.000	0.000
Rhodoblastus cf. sphagnicola	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Rhodococcus cf. equi	0.000	0.000	0.033	0.036	0.000	0.000	0.000
Rhodoplanes cf. elegans	0.000	0.079	0.033	0.072	0.000	0.000	0.000
Rhodopseudomonas cf. faecalis	0.000	0.000	0.000	0.000	0.000	0.000	0.072
Rhodopseudomonas cf. palustris	0.000	0.119	0.033	0.000	0.000	0.000	0.072
Rubritalea cf. spongiae	0.000	1.665	0.000	0.000	0.000	0.000	0.000
Rubrivivax cf. gelatinosus	0.000	0.040	0.000	0.000	0.000	0.000	0.000

Species	*Myco. smithii* 29–02 Worker	*Myco. smithii* 29–02 Garden	*Myco. smithii* 01–03 Worker	*Myco. smithii* 01–03 Garden	*Cypho. wheeleri* 27–01 Worker	*Cypho. wheeleri* 27–01 Garden	*Trachy. septentrionalis* 08–03 Worker
Ruminococcus cf. albus	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Saccharomonospora cf. paurometabolica	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Saccharopolyspora cf. erythraea	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Serinicoccus cf. marinus	0.000	0.000	0.000	0.000	0.000	0.000	2.751
Shinella cf. granuli	0.000	0.119	0.000	0.109	0.000	0.000	0.000
Shinella cf. zoogloeoides	0.000	0.000	0.033	0.978	0.000	0.000	0.000
Sinorhizobium cf. americanum	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Skermania cf. piniformis	0.000	0.000	0.000	0.145	0.000	0.000	0.000
Solibacter cf. usitatus	0.000	0.119	0.000	0.036	0.000	0.000	0.000
Solirubrobacter cf. pauli	0.000	0.000	0.000	0.000	0.000	0.000	0.688
Sphingomonas cf. aquatilis	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Sphingomonas cf. panni	0.027	0.000	0.000	0.036	0.000	0.000	0.000
Sphingopyxis cf. chilensis	0.082	0.357	0.033	0.398	0.000	0.000	0.000
Sphingopyxis cf. witflariensis	0.000	0.000	0.000	0.435	0.000	0.000	0.000
Spiroplasma cf. insolitum	0.027	0.000	0.000	0.000	0.161	0.532	0.000
Spiroplasma cf. syrphidicola	0.549	0.040	0.000	0.000	7.836	15.559	0.000
Staphylococcus cf. aureus	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Staphylococcus cf. capitis	0.000	0.040	0.000	0.000	0.000	0.000	0.036
Stella cf. humosa	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Stenotrophomonas cf. maltophilia	5.934	3.132	0.000	0.000	0.000	0.000	0.000
Streptococcus cf. mitis	0.000	0.040	0.000	0.109	0.000	0.000	0.072
Streptococcus cf. pyogenes	0.000	0.119	0.000	0.000	0.000	0.000	0.000
Streptococcus cf. thermophilus	0.000	0.159	0.000	0.000	0.000	0.000	0.000
Streptomyces cf. cinereoruber	0.000	0.000	0.000	0.036	0.000	0.000	0.000
Streptomyces cf. cinnabarinus	0.027	0.198	0.000	0.000	0.000	0.000	0.000
Streptomyces cf. macrosporus	0.000	0.000	0.000	0.217	0.000	0.000	0.000
Streptomyces cf. olivoreticuli	0.000	0.000	0.000	0.326	0.032	0.000	0.000
Streptomyces cf. resistomycificus	0.000	0.159	0.000	0.000	0.000	0.000	0.000
Streptomyces cf. viridobrunneus	0.000	0.000	0.065	0.833	0.000	0.000	0.000
Terrimonas cf. ferruginea	0.000	0.000	0.000	0.109	0.032	0.000	0.688
Tetrasphaera cf. australiensis	0.000	0.000	0.000	0.000	0.000	0.000	0.036
Thermomonas cf. haemolytica	0.000	0.040	0.000	0.000	0.161	0.000	0.000
thin cf. bent	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Thioalkalivibrio cf. denitrificans	0.000	0.000	0.000	0.290	0.000	0.000	0.000
Tsukamurella cf. tyrosinosolvens	0.137	0.238	0.065	0.471	0.000	0.000	0.326
Variovorax cf. dokdonensis	0.000	0.317	0.000	0.000	0.000	0.000	0.000
Veillonella cf. dispar	0.000	0.000	0.000	0.072	0.000	0.000	0.000
Woodsholea cf. maritima	0.000	0.040	0.000	0.000	0.000	0.000	0.000
Xanthomonas cf. campestris	0.000	0.079	0.000	0.000	0.000	0.000	0.000
Xanthomonas cf. group	0.027	0.000	0.000	0.000	0.000	0.000	0.000
Xenophilus cf. azovorans	0.000	0.000	0.033	0.254	0.000	0.000	0.000

Table S2. Richness and diversity indices for each of the seven bacterial communities screened (OTU = Operational Taxonomic Unit; ACE = Abundance-based Coverage Estimator). Advantages and disadvantages of each index are explained in ref. 1

		OTUs observed			Rarefaction			Chao1 Richness			ACE Richness			Shannon Diversity		
Ant species, sample type	#sequences	1%	3%	5%	1%	3%	5%	1%	3%	5%	1%	3%	5%	1%	3%	5%
M. smithii 29–02, workers	4602	288	95	58	286.5	94.6	57.8	584.1	165.3	82.4	556.8	149.8	81.4	4.22	2.54	2.24
M .smithii 29–02, garden	3643	344	147	100	338.1	145.4	99.1	620.2	176.3	136.1	567.9	177.8	116.9	4.58	3.28	3.09
M. smithii 01–03, workers	3741	290	95	63	281.9	92.8	61.5	482.4	140.8	86.0	487.5	136.8	84.6	4.37	2.55	1.62
M. smithii 01–03, garden	6038	544	219	135	540.2	217.8	134.5	1054.1	334.6	190.5	1001.1	309.6	169.7	4.73	3.46	2.86
C. wheeleri 27–01, workers	5659	220	72	48	219.1	71.7	47.8	331.6	117.1	82.2	347.3	114.9	78.8	3.47	1.71	0.99
C. wheeleri 27–01, garden	4715	119	18	10	117.8	17.7	12.2	163.0	23.6	12.0	148.7	29.4	16.1	2.44	0.27	0.25
T. septentrionalis. 08–03, workers	3431	188	58	46	181.5	56.4	44.8	302.0	85.1	67.0	315.5	87.1	69.3	3.87	2.02	1.85

1. Hughes JB, Hellmann JJ, Ricketts TH, JM Bohannan BJM (2001) Counting the uncountable: Statistical approaches to estimating microbial diversity. *Appl Environ Microbiol* 67:4399–4406.

Table S3. Taxonomic placement and sources of pseudonocardiaceous isolates tested for antifungal activity

Tested species (code)	Source	Source colony ID	Organism	GenBank accession number
*Pseudonocardia*1 (P1)	*Trachymyrmex zeteki* worker	RMMA050816–03	*Pseudonocardia sp.*	FJ948108
*Pseudonocardia*2 (P2)	*Trachymyrmex zeteki* worker	RMMA050818–12	*Pseudonocardia sp.*	FJ948109
*Pseudonocardia*3 (P3)	*Trachymyrmex turrifex* worker	AGH000427–01	*Pseudonocardia sp.*	FJ948110
*Pseudonocardia*4 (P4)	*Trachymyrmex septentrionalis* worker	AMG040508–03	*Pseudonocardia sp.*	FJ948111
*Pseudonocardia*5 (P5)	*Cyphomyrmex wheeleri* worker	UGM030427–01	*Pseudonocardia sp.*	FJ948112
*Amycolatopsis*1 (Amy1)	*Mycocepurus smithii* worker	UGM030329–02	*Amycolatopsis sp.*	FJ948113
*Amycolatopsis*2 (Amy2)	*Mycocepurus smithii* worker	UGM010402–08A	*Amycolatopsis sp.*	FJ948114
Pseudonocardia (PY1)	*Cyphomyrmex wheeleri* worker	UGM030427–01	*Pseudonocardia sp.*	FJ948115
Pseudonocardia (PT1)	*Cyphomyrmex wheeleri* male	UGM030427–01	*Pseudonocardia sp.*	FJ948116
Pseudonocardia (PT1)	*Mycocepurus smithii* worker	UGM010401–03	*Pseudonocardia sp.*	FJ948117
Pseudonocardia (TMWB1)	*Mycocepurus smithii* worker	UGM010401–03	*Pseudonocardia sp.*	FJ948118
Pseudonocardia (BMWB1)	*Mycocepurus smithii* worker	UGM010401–03	*Pseudonocardia sp.*	FJ948119

Table S4. Taxonomic placement and source of test fungi used in antibiotic challenges with pseudonocardiaceous secretions (GP = garden pathogen; AP = attine-ant pathogen; S = saprotroph fungus; EP = endophytic fungus; C = cultivar fungus; GE = general entomopathogen)

Test Fungus	Type	Taxonomic Name, Genbank Aceessions	Order, Family	Source of Test Fungus
Garden Pathogen				
Test 1	GP	*Syncephalastrum racemosum* accessions FJ948130, FJ948146	Mucorales, Syncephalastraceae	Garden of *Trachymyrmex zeteki* (colony RMMA050818–12)
Test 2	GP	*Escovopsis* sp. (cf. *weberi*) accessions FJ948131, FJ948147	Hypocreales, Hypocreaceae	Garden of *Cyphomyrmex wheeleri* (colony UGM030427–01)
Test 23	GP	*Escovopsis* sp. accession FJ948162	Hypocreales, Hypocreaceae	Garden of *Trachymyrmex turrifex* (colony UGM051119–01; RC005)
Test 25	GP	*Escovopsis* sp. (cf. *weberi*) accessions FJ948163	Hypocreales, Hypocreaceae	Garden of *Trachymyrmex zeteki* (colony SES020522–02)
Attine Ant Pathogen				
Test 3	AP	*Simplicillium lanosoniveum* accessions FJ948132, FJ948148	Hypocreales, Cordycipitaceae	Worker cuticle, *Mycocepurus smithii* (colony UGM030329–02)
Test 4	AP	*Fusarium solani* accessions FJ948133, FJ948149	Hypocreales, Hypocreaceae	Diseased queen, *Atta texana* (colony UGM080525–01)
Test 16	AP	*Acrodontium* sp.[1] accessions FJ948141, FJ948158	mitosporic Ascomycota, incertae sedis	Diseased queen, *Acromyrmex versicolor* (colony UGM070721-U)
General Entomopathogen				
5465	GE	*Beauveria bassiana*	Hypocreales, Clavicipitaceae	Overwintering adult, *Galerucella sp.* (Coleoptera: Chrysomelidae)
2575	GE	*Metarhizium anisopliae*	Hypocreales, Clavicipitaceae	*Curculio caryae* (Coleoptera: Curculionidae)
6147	GE	*Beauveria bassiana*	Hypocreales, Clavicipitaceae	Pupa, *Galleria mellonella* (Lepidoptera: Pyralidae)
3288	GE	*Beauveria bassiana*	Hypocreales, Clavicipitaceae	*Spodoptera frugiperda* (Lepidoptera: Noctuidae)
5991	GE	*Beauveria bassiana*	Hypocreales, Clavicipitaceae	Earwig (Dermaptera)
6907	GE	*Beauveria bassiana*	Hypocreales, Clavicipitaceae	*Coptotermes formosanus* (Isoptera: Rhinotermitidae)
Endophyte / Saprotroph				
Test 5	S	*Cyphellophora* sp. accessions FJ948134, FJ948150	Chaetothyriales, Herpotrichiellaceae	Garden of *Trachymyrmex turrifex* (colony AGH000427–01)
Test 7	S	*Eucasphaeria*/*Niesslia* (cf. exilis)[2] accessions FJ948135, FJ948152	Hypocreales incertae sedis	Garden of *Mycocepurus smithii* (colony UGM010402–08A)
Test 18	S/EP	*Acremonium murorum* accessions FJ948143, FJ948160	Hypocreales, mitosporic Hypocreales	Worker of *Cyphomyrmex wheeleri* (colony UGM030427–01)
Test 19	EP/S	*Alternaria tenuissima* accessions FJ948144, FJ948161	Pleosporales, Pleosporaceae	Garden of *Atta texana* (colony UGM070317–04)
Test 17	S/AP	*Verticillium leptobactrum*[3] accessions FJ948142, FJ948159	Hypocreales, mitosporic Hypocreales	Worker of *Trachymyrmex turrifex* (colony AGH000427–01)
Test 27	EP	*Phoma* sp. (cf. glomerata) accessions FJ985694, FJ985695	mitosporic Ascomycota	Garden of *Atta texana* (colony UGM070317–04)
Attine Cultivar				
Test 6	C	*Leucocoprinus* sp. accession FJ948151	Agaricales, Agaricaceae	Garden of *Trachymyrmex turrifex* (colony AGH000427–01)
Test 8	C	*Leucocoprinus* sp. accessions FJ948136, FJ948153	Agaricales, Agaricaceae	Garden of *Mycocepurus smithii* (colony UGM010402–08A)
Test 9	C	*Leucocoprinus* sp. accessions FJ948137, FJ948154	Agaricales, Agaricaceae	Garden of *Trachymyrmex zeteki* (colony RMMA050816–03)
Test 11	C	*Leucocoprinus* sp. accessions FJ948138, FJ948155	Agaricales, Agaricaceae	Garden of *Mycocepurus smithii* (colony UGM030329–02)
Test 13	C	*Leucocoprinus* sp. accessions FJ948139, FJ948156	Agaricales, Agaricaceae	Garden of *Cyphomyrmex wheeleri* (colony UGM030427–01)
Test 15	C	*Leucocoprinus* sp. accessions FJ948140, FJ948157	Agaricales, Agaricaceae	Garden of *Trachymyrmex septentrionalis* (colony AMG040508–03)
Test 22	C	*Leucocoprinus* sp. accession FJ948145	Agaricales, Agaricaceae	Garden of *Trachymyrmex zeteki* (colony RMMA050818–12)

[1]*Acrodontium* is classified here as an attine pathogen because we have repeatedly isolated it from diseased *Acromyrmex* and *Trachymyrmex* queens kept in lab colonies.
[2]Crous et al. (1) discuss the close proximity of *Eucasphaeria* and *Niesslia.*
[3]*Verticillium leptobactrum* is classified here as a saprotroph because it is most frequently isolated from rotting plant material or soil, but less commonly from insect sources.

1. Crous PW, Mohammed C, Glen M, Verkley G, Groenewald JZ (2007) Eucalyptus microfungi known from culture. *Fungal Diversity* 25:19–36.