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

Ruchira Sen[a,1], Heather D. Ishak[a], Dora Estrada[a], Scot E. Dowd[b], Eunki Hong[a], and Ulrich G. Mueller[a,1]

[a]Section of Integrative Biology, University of Texas, Austin, TX 78712; and [b]Medical Biofilm Research Institute, 4321 Marsha Sharp Freeway, Lubbock, TX 79407

Edited by Raghavendra Gadagkar, Indian Institute of Science, Bangalore, India, and approved August 14, 2009 (received for review May 1, 2009)

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 non-adaptive 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

Author contributions: R.S., H.D.I., E.H., and U.G.M. designed research; R.S., H.D.I., D.E., S.E.D., E.H., and U.G.M. performed research; S.E.D. and U.G.M. contributed new reagents/analytic tools; R.S., H.D.I., S.E.D., and U.G.M. analyzed data; and R.S., H.D.I., and U.G.M. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. FJ948108-FJ948163, FJ985694-FJ985695, SRA008625.9).

See Commentary on page 17611.

[1]To whom correspondence should be addressed. E-mail: ruchira05@gmail.com or umueller@mail.utexas.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0904827106/DCSupplemental.

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).

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 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).

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

These 2 *Pseudonocardia* species were also found in workers from 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.

ACKNOWLEDGMENTS. We thank A. Rodrigues for mycological identification; S. Rehner for entomopathogens; P. Abbott, C. Currie, R. Gadagkar, B. Klein, N. Mehdiabadi, S. Mikheyev, C. Rabeling, A. Rodrigues, S. H. Yek, and 3 anonymous reviewers for constructive comments; F. Denison for first suggesting that attine actinomycetes may harm the ant-cultivars; and the Autoridad Nacional del Ambiente (ANAM) de Panamá for collecting permits. This work was supported by National Sceince Foundation Award DEB-0639879 to UGM and a Research Fellowship to EH from the University of Texas, Austin, TX.

1. Mueller UG, Gerardo N, Schultz TR, Aanen D, Six DL (2005) The evolution of agriculture in insects. *Ann Rev Ecol Evol Sys* 36:563–595.
2. Bacci M, Ribeiro SB, Casarotto MEF, Pagnocca FC (1995) Biopolymer-degrading bacteria from nests of the leaf-cutting ant *Atta sexdens rubropilosa*. *Braz J Med Biol Res* 28:79–82.
3. Carreiro SC, et al. (1997) Yeasts associated with the nests of the leaf-cutting ant *Atta sexdens rubropilosa* Forel, 1908. *Antonie van Leeuwenhoek* 71:243–248.
4. Santos AV, Dillon RJ, Dillon VM, Reynolds SE, Samuels RI (2004) Occurrence of the antibiotic producing bacterium *Burkholderia sp.* in colonies of the leaf-cutting ant *Atta sexdens rubropilosa*. *FEMS Microbiol Lett* 239:319–323.
5. Currie CR (2001) A community of ants, fungi, and bacteria: A multilateral approach to studying symbiosis. *Annu Rev Microbiol* 55:357–380.
6. Rodrigues A, Bacci M, Mueller UG, Ortiz A, Pagnocca FC (2008) Microfungal "weeds" in the leafcutter ant symbiosis. *Microb Ecol* 56:604–614.
7. Rodrigues A, Carletti CD, Bueno OC, Pagnocca FC (2008) Leaf-cutting ant faecal fluid and mandibular gland secretion: Effects on microfungi spore germination. *Braz J Microbiol* 39:64–67.
8. Rodrigues AR, Cable N, Mueller UG, Bacci M, Pagnocca FC (2009) Antagonistic interactions between garden yeasts and microfungal garden pathogens of leaf-cutting ants. *Antonie van Leeuwenhook* 6:331–342.
9. Currie CR, Mueller UG, Malloch D (1999) The agricultural pathology of ant fungus gardens. *Proc Natl Acad Sci USA* 96:7998–8002.
10. Rodrigues A, Pagnocca FC, Bueno OC, Pfenning LH, Bacci M (2005) Assessment of microfungi in fungus gardens free of the leaf-cutting ant *Atta sexdens rubropilosa* (Hymenoptera: Formicidae). *Sociobiology* 46:329–334.
11. Reynolds HT, Currie CR (2004) Pathogenicity of *Escovopsis weberi*: The parasite of the attine ant-microbe symbiosis directly consumes the ant-cultivated fungus. *Mycologia* 96:955–959.
12. Currie CR, Scott JA, Summerbell RC, Malloch D (2003) Corrigendum: Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 423:461.
13. Cafaro MJ, Currie CR (2005) Phylogenetic analysis of mutualistic filamentous bacteria associated with fungus-growing ants. *Can J Microbiol* 51:441–446.
14. Currie CR, Poulsen M, Mendenhall J, Boomsma JJ, Billen J (2006) Coevolved crypts and exocrine glands support mutualistic bacteria in fungus-growing ants. *Science* 311:81–83.
15. Haeder S, Wirth R, Herz H, Spiteller D (2009) Candicidin-producing *Streptomyces* support leaf-cutting ants to protect their fungus garden against the pathogenic fungus *Escovopsis*. *Proc Natl Acad Sci USA* 106:4742–4746.
16. Poulsen M, Bot NM, Currie CR, Nielsen MG, Boomsma JJ (2003) Within-colony transmission and the cost of a mutualistic bacterium in the leaf-cutting ant *Acromyrmex octospinosus*. *Funct Ecol* 17:260–269.
17. Mueller UG, Dash D, Rabeling C, Rodrigues A (2008) Coevolution between attine ants and actinomycete bacteria: A reevaluation. *Evolution (Lawrence, Kans)* 62:2894–2912.
18. Currie CR, Scott JA, Summerbell RC, Malloch D (1999) Fungus-growing ants use antibiotic-producing bacteria to control garden parasites. *Nature* 398:701–704.
19. Currie CR, et al. (2003) Ancient tripartite coevolution in the attine antmicrobe symbiosis. *Science* 299:386–388.
20. Poulsen M, Erhardt DP, Molinaro DJ, Ting-Li L, Currie CR (2007) Antagonistic bacterial interactions help shape host-symbiont dynamics within the fungus-growing ant-microbe mutualism. *PLoS ONE* 2:e960.
21. Youngstaedt E (2008) All that makes fungus gardens grow. *Science* 320:1006–1007.
22. Suen G, Currie CR (2008) Ancient fungal farmers of the insect world. *Microbiol Today* 35:172–175.
23. Poulsen M, Cafaro M, Boomsma JJ, Currie CR (2005) Specificity of the mutualistic association between actinomycete bacteria and two sympatric species of *Acromyrmex* leaf-cutting ants. *Mol Ecol* 14:3597–3604.
24. Little AEF, Currie CR (2008) Indirect interaction web reveals how black yeast symbionts compromise the efficiency of antibiotic defenses in fungus-growing ants. *Ecology* 89:1216–1222.
25. Goodfellow M, Williams ST (1983) Ecology of actinomycetes. *Annu Rev Microbiol* 37:189–216.
26. Embley, TM (1992) in *The Prokaryotes*, eds Balows A, Truper HG, Dworkin M, Harder W, Schleifer KH (Springer Verlag, Berlin), pp 996–1027.
27. Poulsen M, Currie CR (2006) in *Insect Symbiosis*, eds Bourtzis K, Miller TA (Boca Raton, Florida), pp 57–77.
28. Frank SA (1996) Host-symbiont conflict over the mixing of symbiotic lineages. *Proc R Soc Lond Ser B* 263:339–344.
29. Schultz TR, Mueller UG, Currie CR, Rehner SA (2005) in *Insect-Fungal Associations: Ecology and Evolution*, eds Vega F, Blackwell M (Oxford Univ Press, New York), pp 149–190.
30. Currie CR (2004) in *Symbiosis: Mechanisms and Model Systems*, ed Seckbach J (Kluwer Academic Publishers, New York) pp 687–699.
31. Holzmann D (2006) Bacteria joined with ants in symbiosis that later added cultivated fungi. *Microbe* 1:106–107.
32. Kumar H, Patole MS, Shouche YS (2006) Fungal farming: A story of four partner evolution. *Curr Sci* 90:1463–1464.
33. Diamond J, Zimmer C, Evans EM, Allison L, Disbrow S (2006) *Virus and the Whale: Exploring Evolution in Creatures Small and Large* (National Science Teachers Association Press, Arlington, VA).
34. Sanchez-Pena SR, Sánchez-Ovalle MR, Gallegos-Morales G, Sánchez-Arizpe A (2008) In vitro antagonism of actinomycetes isolated from fungus-growing ants against plant pathogenic fungi. *Phytoparasitica* 36:322–325.
35. Oh D, Poulsen M, Currie CR, Clardy J (2009) Dentigerumycin: A bacterial mediator of an ant-fungus symbiosis. *Nat Chem Biol* 5:391–393.
36. Kost C, et al. (2007) Non-specific association between filamentous bacteria and fungus-growing ants. *Naturwissenschaften* 94:821–828.
37. Currie CR, Bot ANM, Boomsma JJ (2003) Experimental evidence of a tripartite mutualism: Bacteria protect ant fungus gardens from specialized parasites. *Oikos* 101:91–102.
38. Little AEF, Currie CR (2007) Symbiont complexity: Discovery of a fifth symbiont in the attine ant-microbe symbiosis. *Biol Lett* 3:501–504.
39. Little AEF, Murakami T, Mueller UG, Currie CR (2006) Defending against parasites: Fungus-growing ants combine specialized behaviours and microbial symbionts to protect fungus gardens. *Biol Lett* 2:12–16.
40. Mikheyev AS, Vo T, Mueller UG (2008) Phylogeography of post-Pleistocene population expansion in a fungus-gardening ant and its microbial mutualists. *Mol Ecol* 17:4480–4488.
41. Fernández-Marín H, Zimmerman JK, Nash DR, Boomsma JJ, Wcislo WT (2009) Reduced biological control and enhanced chemical pest management in the evolution of fungus farming in ants. *Proc R Soc Lond Ser B* 276:2263–2269.
42. Baker GC, Smith JJ, Cowan DA (2003) Review and re-analysis of domain-specific 16S primers. *J Microbiol Methods* 55:541–555.
43. Morón R, González I, Genilloud O (1999) New genus-specific primers for the PCR identification of members of the genera *Pseudonocardia* and *Saccharopolyspora*. *Int J Syst Bacteriol* 49:149–162.
44. Dowd SE, et al. (2008) Survey of bacterial diversity in chronic wounds using Pyrosequencing, DGGE, and full ribosome shotgun sequencing. *BMC Microbiol* 8:43.