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COEVOLUTION BETWEEN ATTINE ANTS AND ACTINOMYCETE BACTERIA: A REEVALUATION

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We reassess the coevolution between actinomycete bacteria and fungus-gardening (attine) ants. Actinomycete bacteria are of special interest because they are metabolic mutualists of diverse organisms (e.g., in nitrogen-fixation or antibiotic production) and because *Pseudonocardia* actinomycetes are thought to serve disease-suppressing functions in attine gardens. Phylogenetic information from culture-dependent and culture-independent microbial surveys reveals (1) close affinities between free-living and ant-associated *Pseudonocardia*, and (2) essentially no topological correspondence between ant and *Pseudonocardia* phylogenies, indicating frequent bacterial acquisition from environmental sources. Identity of ant-associated *Pseudonocardia* and isolates from soil and plants implicates these environments as sources from which attine ants acquire *Pseudonocardia*. Close relatives of *Atta* leafcutter ants have abundant *Pseudonocardia*, but *Pseudonocardia* in *Atta* is rare and appears at the level of environmental contamination. In contrast, actinomycete bacteria in the genera *Mycobacterium* and *Microbacterium* can be readily isolated from gardens and starter-cultures of *Atta*. The accumulated phylogenetic evidence is inconsistent with prevailing views of specific coevolution between *Pseudonocardia,* attine ants, and garden diseases. Because of frequent acquisition, current models of *Pseudonocardia*-disease coevolution now need to be revised. The effectiveness of *Pseudonocardia* antibiotics may not derive from advantages in the coevolutionary arms race with specialized garden diseases, as currently believed, but from frequent recruitment of effective microbes from environmental sources. Indeed, the exposed integumental structures that support actinomycete growth on attine ants argue for a morphological design facilitating bacterial recruitment. We review the accumulated evidence that attine ants have undergone modifications in association with actinomycete bacteria, but we find insufficient support for the reverse, modifications of the bacteria resulting from the interaction with attine ants. The defining feature of coevolution—reciprocal modification—therefore remains to be established for the attine ant-actinomycete mutualism.

KEY WORDS: ***Atta*, Attini, fungus-growing ants, mutualism, *Pseudonocardia*, symbiosis.**

Coevolution, the reciprocal evolutionary influence between species, is a key biological process. All species interact with other lineages, leading often to the reciprocal, selective interplay that defines coevolution. Coevolutionary processes have traditionally been classified along a continuum ranging from specific coevolution (also called one-to-one, strong, or tight coevolution; e.g., host-specific parasites) to diffuse coevolution (many-to-many, loose, multispecies, or guild coevolution; e.g., between a guild of plant species and a guild of pollinators) (Janzen 1980; Futuyma and Slatkin 1983; Futuyma 1998). Although diffuse coevolutionary processes are by definition more amorphous, they clearly shape ecological communities because of their ubiquity and immense

ecological impacts. In fact, it has been more difficult to find convincing cases of specific coevolution than diffuse coevolution, such as the diffuse interactions between plants and pollinators, predators and prey, plants and rhizobia bacteria, fungi and algae in lichens, and polyps and algae in corals (Futuyma and Slatkin 1983; Thompson 2005).

Coevolution between fungus-growing (attine) ants and their various microbial associates was historically thought to be a case of specific coevolution (Chapela et al. 1994; Hinkle et al. 1994; Currie et al. 1999b). This view of one-to-one coevolution was based on the observation that specific microbes, such as the cultivated fungi or integument-inhabiting bacteria in the genus *Pseudonocardia*, are vertically transmitted from one generation to the next by dispersing queens. This intergenerational transfer mechanism led to the expectation of long-lasting associations, codiversification, and eventual phylogenetic cocladogenesis between ant and microbial associates. The first tests of cocladogenesis in the attine ant–microbe symbiosis seemed to corroborate this view when phylogenetic analyses revealed patterns of clade-to-clade correspondences between the ants and their microbial associates (Chapela et al. 1994; Hinkle et al. 1994; Currie et al. 2003c). However, later comprehensive analyses invalidated these early interpretations of attine ant–microbe coevolution, revealing significant diffuse coevolution and frequent lineage–lineage reassociations over both evolutionary and ecological time (Mueller et al. 1998; Villesen et al. 2004; Gerardo et al. 2004, 2006b; Mikheyev et al. 2006; Gerardo and Caldera 2007).

To further characterize the coevolutionary interactions between microbes and attine ants, we conducted culture-dependent and culture-independent (PCR-based) surveys of the microbes in the gardens of attine ants, as well as the microbial inocula carried by foundress queens when they disperse from natal colonies to found their independent gardens. We summarize here our results pertaining to the actinomycete bacteria (class Actinobacteria, order Actinomycetales; results on other microbes will be presented elsewhere), integrating published phylogenetic information with our new information on other attine-associated actinomycete bacteria. We emphasize here the associations with actinomycete bacteria because such bacteria are known to be particularly rich producers of bioactive compounds (Goodfellow and Williams 1983; Embley 1992), and because they have been implicated as disease-suppressing agents in nests of fungus-growing ants (Currie et al. 1999b; Little et al. 2003, 2006; Mangone and Currie 2007) and digger wasps (Kaltenpoth et al. 2005, 2006; Goettler et al. 2007; Kaltenpoth and Strohm 2007). Our microbial surveys are incomplete in that they do not cover the full range of attine genera, in that only a portion of the associated microbes is screened, and in that they are qualitative rather than quantitative (the surveys aimed at identification of prominent bacterial associates, but not at estimation of absolute abundances). Despite these limitations, the integration of our new findings with published information now prompts conceptual revision of the current model of attine ant–actinomycete coevolution.

To understand the emerging insights in the context of previous conclusions, we first review the accumulated evidence that led to prevailing beliefs about attine ant–microbe coevolution.

ATTINE ANT–MICROBE COEVOLUTION

Three kinds of attine microbes have been studied in detail: (1) the cultivated fungi, (2) *Escovopsis* fungi that parasitize the cultivated fungi, and (3) actinomycete bacteria growing on the integument of the ants (Currie 2001a; Mueller et al. 2005; Schultz et al. 2005; Poulsen and Currie 2006). A plethora of additional microbes occur in attine gardens, including antibiotic-producing *Burkholderia* bacteria (Santos et al. 2004), cellulose-degrading bacteria (Bacci et al. 1995), and yeasts (Carreiro et al. 1997). The functional roles of these secondary microbes in the attine gardens and their possible coevolutionary interactions with the ants are not understood, and they are therefore not reviewed here.

Ant-cultivar coevolution

Attine fungiculture originated about 50 million years ago when the ancestral attine ants evolved the ability to sustain the growth of leaflitter-decomposing fungi. The original attine most likely was a debris-collector, accumulating plant detritus as substrate for fungal growth in their nests, such as dried leaf bits, withering flower parts, seeds, or arthropod feces (Schultz and Brady 2008; Mueller and Rabeling 2008). This ancestral form of fungiculture is still found in the most basal attine genera, which are debris-collecting fungiculturists whose fungi retain close population-genetic ties to free-living fungal populations (Basidiomycota: Lepiotaceae, Leucocoprineae). The close population-genetic ties indicate either that novel fungal strains are regularly imported into the symbiosis from free-living fungal populations, or that cultivated strains occasionally escape from the symbiosis to revert to feral life, or both (Mueller et al. 1998, 2005; Vo et al. 2008). One key evolutionary transition in attine fungiculture was the shift from this open fungicultural system of the primitive (lower) attine ants to the more closed system of higher attine ants. In this derived system (higher-attine fungiculture), domesticated cultivars are thought to exist disjoint from free-living relatives, tightening the coevolutionary process through more specialized, closed fungiculture (Mueller et al. 2005; Schultz and Brady 2008). A later, even narrower specialization was the fungiculture of leafcutting ants, which originated about 8–12 million years ago with a transition to a highly specialized fungus (leafcutter fungus; Wirth et al. 2003; Mikheyev et al. 2006; Schultz and Brady 2008). Even though higher-attine and leafcutter fungiculture is more specialized, ant-cultivar lineage–lineage reassociation occurs frequently within each group. For example, leafcutting ants comprise a clade of at

least 50 species (genera *Acromyrmex* and *Atta*) but they cultivate largely a single species of fungus, in a many-to-one coevolutionary relationship between ant and fungal species (Mikheyev et al. 2006, 2007; Mueller et al., unpubl. ms.). Moreover, most leafcutter species regularly trade cultivar lineages between each other through unknown exchange mechanisms (Bot et al. 2001a; Mikheyev et al. 2006, 2007; U. G. Mueller, S. Bruschi, H. D. Ishak, S. E. Solomon, A. S. Mikheyev, and M. Bacci, unpubl. ms.).

At the origin of attine fungiculture, the ants also evolved the ability to transmit fungi between generations, thus tightening the coevolutionary association by vertical transmission of fungi. Vertical, fungal transmission is achieved by dispersing queens, each carrying a pellet of compacted garden mycelium and substrate in a specialized pocket in the mouth (the infrabuccal pocket) and using it as a starter culture for a new garden in the incipient nest (Ihering 1898; Huber 1905). Pellets of leafcutter queens are large (about 0.5–0.7 mm diameter in *Atta* queens), and they can be extracted experimentally to examine the contents (Huber 1905; Quinlan and Cherrett 1978; Febvay and Kermarrec 1981). Pellets of leafcutter queens contain a diversity of microbes (Wheeler and Bailey 1920; Quinlan and Cherrett 1978; Febvay and Kermarrec 1981), but the secondary, noncultivar microbes in queen-pellets have not been identified. The presence of actinomycete bacteria in the pellets of attine workers (Little et al. 2003, 2006) raised the question whether queen-pellets may also contain actinomycetes that may be vertically transmitted between ant generations, prompting our study to profile the actinomycete microbes present in the queen-pellets of the leafcutter ant *Atta texana*.

Ant-cultivar-Escovopsis *coevolution*

Gardens of attine ants contain a diversity of detrimental microbes (Fisher et al. 1996; Currie et al. 1999a; Rodrigues et al. 2004, 2005, 2008a). The best studied of these are filamentous fungi in the genus *Escovopsis* (Ascomycota: Hypocreales), which appear to exist as hyphal parasites of the cultivated fungi (Currie et al. 1999a; Reynolds and Currie 2004; Taerum et al. 2007), but can also persist as spores in attine gardens (Rodrigues et al. 2004). *Escovopsis* attacks gardens of most attine lineages, suggesting a long coevolutionary history between attine ants, their cultivars, and the *Escovopsis* garden parasites. Consistent with this view, attine ants and their cultivated fungi have evolved diverse defenses, such as garden grooming and weeding by the ants, as well as chemical defenses of both ants and cultivar against *Escovopsis* (Bot et al. 2001b; Currie and Stuart 2001; Fernández-Marín et al. 2003; Rodrigues et al. 2004; Fernández-Marín 2006a; Gerardo et al. 2006a,b; Rodrigues et al. 2008b). In addition, Currie et al. (2003c) reported a pattern of cocladogenesis between these three interacting groups. However, more recent analyses revealed several exceptions to this pattern of cocladogenesis (Gerardo et al. 2006b; N. Gerardo, pers. comm.). Specifically, the most abundant *Escovopsis* in gardens of the primitive attine genus *Apterostigma* (the so-called brown-spored *Escovopsis*) are very closely related to *Escovopsis* prevalent in gardens of leafcutter ants (Gerardo et al. 2006b), one of the most highly derived clades of attine ants that cultivate very different fungi than *Apterostigma* (Munkacsi et al. 2004), indicating less specificity in association than previously believed (Currie et al. 2003c).

Ant-Escovopsis-Pseudonocardia *coevolution*

Many attine ants carry on their bodies conspicuous whitish coatings. Originally thought to be an inert waxy secretion or mineral accretion (Wheeler 1911; Creighton 1950; Cole 1952; Weber 1972), these coatings were later shown to contain antibiotic-producing bacteria in the order Actinomycetales (Currie et al. 1999b; Cafaro and Currie 2005). The proportion of live versus inert matter in these integumental accumulations is still unknown. Different attine ant taxa carry the predominant accumulations on taxon-specific locations. The more primitive attine genera generally show the greatest accumulation on the venter between the legs, whereas a derived clade (the higher attines, plus the *Cyphomyrmex* species in the *costatus*-group) shows the predominant accumulation on a specialized surface on the antero-ventral side of the thorax (the propleural plate; Currie et al. 2006). There are many exceptions to this general difference in integumental accumulations between primitive and derived attine lineages (e.g., predominant accumulations on the gaster in some primitive attines; absence of accumulations in some desert-dwelling attines; U. Mueller, pers. obs.). Attine workers are actinomycete-free when eclosing, but begin to accumulate integumental growth within the first few days during interactions with nest mates and garden material (Poulsen et al. 2003a). Further proliferation of the actinomycete accumulation is dependent on worker caste (garden workers generally carry more extensive accumulations than foragers, the smallest workers carry less than larger workers in a garden; Currie et al. 1999b, 2003a; Poulsen et al. 2002) and dependent on disease status of the garden (*Acromyrmex* workers of *Escovopsis*-infected colonies have greater accumulations than uninfected colonies; Currie 2001b; Currie et al. 2003a), suggesting that the ants can regulate the growth of bacteria to modulate antibiotic production. Indeed, the integumental bacteria produce antibiotics effective against *Escovopsis* (Currie et al. 1999b, 2006), and the cuticle underlying the bacterial growth is permeated by glandular ducts that are thought to supply nutrients to the bacterial populations (Currie et al. 2006).

The bacterium in the ant coating was originally identified as a type of *Streptomyces* (Currie et al. 1999b), an actinomycete genus well known for its antibiotic secretions. The extremely slow growth rate of the ant-associated bacterium was unusual for a *Streptomyces*, but biochemical characters placed it within the

taxonomic concept of *Streptomyces* sensu *lato* (R. Summerbell, pers. comm.). Later sequencing information prompted a reclassification into the actinomycete genus *Pseudonocardia* (Currie et al. 2003b; Cafaro and Currie 2005). Assuming that the cuticular bacteria are *Streptomyces*, Kost et al. (2007) surveyed cuticular bacteria of *Acromyrmex octospinosus* (the species studied most intensively by Currie et al. 1999b), trying to replicate as closely as possible Currie et al.'s approach, with the exception of the use of a more nutrient-rich isolation medium (favoring more fast-growing actinomycetes) than the minimum-nutrient medium used by Currie et al. to favor slow-growing actinomycetes (including *Pseudonocardia*). (The reason for Kost et al.'s choice of a different growth medium was that Currie et al.'s [1999] isolation methods were not published in the original study). Kost et al. isolated a significant diversity of actinomycete bacteria (between 1 and 7 strains per colony; and 1 and 3 strains per single worker), including *Streptomyces* strains. Most of these isolates showed activity against *Escovopsis*, and the level of anti-*Escovopsis* activity of attine actinomycetes was slightly lower than the corresponding activity of actinomycetes isolated from workers of two nonattine ant species (Kost et al. 2007). The high level of activity of nonattine actinomycetes against *Escovopsis* was surprising and suggested that the attine actinomycetes are not particularly derived (i.e., not necessarily coevolved with *Escovopsis*), or that *Escovopsis* is relatively easy to suppress, or both. More importantly, the diversity of attine actinomycetes within nests and on single ants suggested to Kost et al. that these bacteria are "dynamically acquired" (e.g., from soil-dwelling populations), rather than strictly vertically inherited from mother to offspring, which Kost et al. predicted would lead to single-strain association due to gradual erosion of microbial diversity over time (lineage sorting).

Even though the bacterial surveys of Currie et al. and Kost et al. are not directly comparable because of the different isolation methods used, the presence of diverse non-*Pseudonocardia* strains on the integument of attine ants implies that Currie et al.'s (1999b, 2003b, 2006) conclusion of long-term, specific *Escovopsis–Pseudonocardia* coevolution may have been premature and that the integumental growth perhaps consists of a microbial community, of which *Pseudonocardia* may be a dominant component. Following this line of thinking, Kost et al. speculated that the integumental microbial community may even undergo ecological turnover, and that novel strains could be regularly recruited from free-living bacterial populations, paralleling the open system that permits regular recruitment of microbes in many other symbioses (e.g., gut bacteria, rhizobia of plants, algae of corals, etc). However, this hypothesis of continuous recruitment of *Pseudonocardia* and other microbial associates seems inconsistent with Poulsen et al.'s (2005) report of only a single *Pseudonocardia* strain on all workers within the same attine nest. Specifically, Poulsen et al. (2005) profiled several workers each from 16 *Acromyrmex* nests [between two and four workers for each of 15 nests (average of 2.5 workers per nest); 24 workers for one additional nest], and found no within-nest genetic variation in EF-Tu PCR products amplified with *Pseudonocardia*-specific primers, but substantial sequence variation of *Pseudonocardia* strains between nests. Given the observed sequence variation, the *Pseudonocardia* populations carried by workers of a single *Acromyrmex* colony appeared to be dominated by a single *Pseudonocardia* strain, but the possible coexistence of this dominant strain with a second minor *Pseudonocardia* strain of maximally 4% representation could not be ruled out (Poulsen et al. 2005). It is possible that this estimate of maximally 4% secondary strains is an underestimate, as PCR/sequencing failures due to multiple *Pseudonocardia*-templates are not included in this analysis. Moreover, because of the use of *Pseudonocardia*-specific primers in this study, the coexistence of *Pseudonocardia* with other actinomycetes in the cuticular accumulation cannot be excluded.

Kost et al.'s hypothesis of bacterial recruitment and nonspecific association is supported by two studies that documented microbial diversity among the cuticular microbes on single ant workers. First, Little and Currie (2007, 2008) documented the presence of black-yeasts within the cuticular coating. These yeasts are thought to parasitize the ant–*Pseudonocardia* mutualism by infiltrating the coating and subsisting on the nutrients provided by the ants for the integumental actinomycetes. Second, in their original phylogenetic analysis of the ant-associated *Pseudonocardia* strains, Cafaro and Currie (2005) actually reported the isolation of diverse microbes from attine integuments. Following general microbiological practice, Cafaro and Currie (2005) identified a subset of these isolates by morphotyping and sequencing two or three representatives for each dominant morphotype (*Pseudonocardia*) associated with three genera of attine ants. The fraction of main morphotype recovered for each of three ant genera was 74% (from *Acromyrmex* ants), 46% (from *Trachymyrmex* ants), and 74% (from *Apterostigma* ants). The remaining 26%, 54%, and 26% of the respective nondominant isolates were not further identified, but likely included a mix of actinomycetes, other bacteria, and perhaps yeasts. The accumulated results suggest that a significant portion of microbial isolates from attines ants (e.g., more than 50% of actinomycete isolates from *Trachymyrmex*) may not be *Pseudonocardia*, supporting Kost et al.'s view that the integumental microbes represent a community, of which components may be dynamically acquired from environmental sources.

Further support for Kost et al's recruitment hypothesis derives from phylogenetic analyses of ant-associated and a few free-living *Pseudonocardia* (Cafaro and Currie 2005; Poulsen et al. 2007). Several soil-dwelling *Pseudonocardia* are nested within the clades of ant-associated *Pseudonocardia*, and distantly related ant lineages may be associated with very closely related

Pseudonocardia lineages. Cafaro and Currie (2005) and Poulsen et al. (2005, 2007) interpret the latter pattern as a result of horizontal transfer between attine lineages. However, if free-living representatives have so far been underrepresented in analyses, then Kost et al's hypothesis of actinomycete recruitment is also consistent with the published phylogenetic relationships (i.e., the published monophylies of ant-associated *Pseudonocardia* clades would then be paraphyletic, because free-living *Pseudonocardia* strains were insufficiently represented in phylogenetic analyses). *Pseudonocardia* recruitment from environmental sources was also one possible interpretation of phylogeographic patterns of the *Pseudonocardia* associated with the higher-attine ant *Trachymyrmex septentrionalis* (Mikheyev et al. 2008), which lives largely allopatric from other higher-attine ants and therefore is unable to acquire *Pseudonocardia* from other attine species through most of its range (Rabeling et al. 2007). Specifically, phylogeographic structures of *T. septentrionalis* and associated *Pseudonocardia* are not correlated, indicating that, even if ant-*Pseudonocardia* genotype pairings are perpetuated through vertical transmission by dispersing queens, these associations appear ephemeral, possibly because of frequent *Pseudonocardia* recruitment from environmental sources (Mikheyev et al. 2008).

The strongest evidence against Kost et al.'s recruitment hypothesis derives from Currie et al.'s (1999b) original study reporting that ant-associated *Pseudonocardia* produce antibiotics that are specifically effective against *Escovopsis* parasites, but that they do not produce antibiotics effective against other filamentous fungi that attine ants may also encounter in their nest environment, such as entomopathogenic, saprophytic, or endophytic fungi. Because actinomycetes in the family *Pseudonocardiaceae* produce a great diversity of potent antifungal and antibacterial compounds (such as the antibacterials erythromycin, vancomycin, and rifampicin; or antifungal polyketides related to nystatin and amphotericin; Embley 1992; Morón et al. 1999; Lazzarini et al. 2000; Lee et al. 2006) and because microbes generally produce cell–cell signaling molecules with accidental antimicrobial side effects (Davies 2006; Yim et al. 2007; Fajardo and Martinez 2008), Kost et al.'s hypothesis of frequent recruitment from environmental sources would predict that *Pseudonocardia* also exhibit activities against at least some fungi other than *Escovopsis*, which was not the case in the original study (Currie et al. 1999b) that concluded narrow activity against *Escovopsis* only.

Because the narrow antibiotic activity has been cited widely as evidence for specific coevolution between ant-associated *Pseudonocardia* and attine ants (Currie et al. 1999b, 2003c; Price et al. 2003; Currie 2004; Mueller et al. 2005; Schultz et al. 2005; Poulsen and Currie 2006), it is worth reviewing the details of this key finding. First, only a single *Pseudonocardia* strain from a worker of *A. octospinosus* was tested (Currie et al. 1999b; C. R. Currie, pers. comm.). This single strain lacked detectable inhibitory effects against 17 tested microfungi, including fungi commonly used for antibiotic screening, entomopathogenic fungi, generalist saprotrophic fungi, and several non-*Escovopsis* fungi isolated from attine nests. In contrast, the tested *Pseudonocardia* strain was antibiotically effective against any *Escovopsis* challenged, including *Escovopsis* isolated from nests of distantly related attine lineages. More recent analyses showed that many ant-associated *Pseudonocardia* do not have a comparable activity against all *Escovopsis*, and that some ant-associated *Pseudonocardia* are completely ineffective against all tested *Escovopsis* (Gerardo 2006; N. Gerardo and M. Poulsen, pers. comm.), indicating that the single *Pseudonocardia* isolate used originally by Currie et al. (1999b) may not be representative for the ant-associated *Pseudonocardia* at large. Moreover, Poulsen et al. (2007) recently showed that *Pseudonocardia* secrete highly effective antibacterial compounds against other *Pseudonocardia* strains, making specific antibiosis only against *Escovopsis* even more remarkable, as many antibacterial compounds also have antifungal properties (e.g., Cowan 1999; Strobel and Daisy 2003) and as microbes generally produce cell–cell signaling molecules with accidental antifungal side effects (e.g., Davies 2006; Yim et al. 2007; Fajardo and Martinez 2008).

To expand on this previous work and to assess the coevolutionary interactions between attine ants and actinomycete bacteria, we conducted culture-dependent and culture-independent screens of microbes in queen pellets and gardens of attine ants.

Materials and Methods

We profiled actinomycete bacteria in queen-pellets of *A. texana* and in gardens of seven representative fungus-growing ant species (seven genera).

PELLET COLLECTION

Infrabuccal pellets were extracted from unmated queens collected on 15 May 2004 ($n = 6$ pellets), 9 May 2005 ($n = 7$), and 9 May 2006 ($n = 7$) from two *A. texana* nests located at Brackenridge Field Laboratory at the University of Texas, Austin, Texas. In addition, seven pellets were extracted from unmated queens collected on 1 May 2006 from an *A. texana* nest at Hornsby Bend Environmental Research Center, Austin, Texas. Mating flights of *A. texana* occur just before dawn on days following heavy spring rains. In preparation for flights, hundreds of male and female alates assemble on their natal nest mounds during the night, and a large number of unmated females can be collected in short time by picking them directly from mounds. Females were collected into large containers, brought immediately to the laboratory, and kept in the dark until pellet extraction. Females rested calmly in the dark container and were not given any food, thus minimizing potential contamination of their pellets. To immobilize females

for pellet extraction, females were briefly chilled by transferring them individually to 5-dram vials and placing each vial on ice for about 3–5 min. Pellets were expelled from chilled queens by pulling on the glossa with a sterilized forceps. Overextension of the glossa evaginates the infrabuccal pocket and expels the pellet, which can then be picked up with a second sterilized forceps for transfer into sterile buffer. Pellets were discarded if they accidentally touched any hair or any mouthpart (other than the evaginated infrabuccal pocket), and only uncontaminated pellets that were expelled cleanly were processed further. Pellets were collected within 4 h of the mating flight, as pellets become discolored with longer time and as laboratory-maintained females naturally discard the pellet during the first 24 h. The compacted mycelium in fresh pellets (within half a day of the mating flight) gives them a light cream-colored appearance, but chlorophyll-containing leaf bits or other unidentified plant matter are also incorporated in each pellet (this plant matter presumably helps sustain the mycelium during the mating flight and during garden initiation). Pellets extracted from queens were macerated in buffer with a sterilized needle, then vortexed for 5 min (in 2004 and 2005) or 10 min (in 2006) to fully disperse and suspend the pellet contents. In the repeat isolations in 2004–2006, different suspension buffers were used to increase diversity of isolated microbes [in 2004: potato-dextrose broth (Difco, Becton, Dickinson & Co. Maryland); in 2005: M9-saline buffer (1.28% Na_2HPO_4, 0.3% KH_2PO_4, 0.05% NaCl, 0.1% NH_4Cl_2); in 2006: peptone buffer (0.2% peptone solution supplemented with 0.05% Tween 80)].

GARDEN COLLECTION

Attine gardens of laboratory colonies were stabilized by removing all substrate from the ants' foraging chambers, then "starving" the gardens for one week (i.e., by not providing substrate). During this week, no new substrate could be added by workers to the gardens, garden surfaces became richly suffused with mycelium, the expanding fungus began to digest the substrate, and communities of secondary microbes presumably became dominated by autochthonous components rather than by transient components introduced accidentally with any substrate. We sampled gardens of queenright colonies of the lower-attine ants *Apterostigma dentigerum*, *Cyphomyrmex costatus*, *C. longiscapus*, and *C. muelleri*; from the higher-attine ants *Sericomyrmex amabilis*; and from the leafcutter ants *Atta colombica*, *A. sexdens*, *A. cephalotes*, and *Acromyrmex coronatus*. These colonies had been collected between 2001 and 2004 in the Republic of Panamá (exact collection location are listed in the Supporting Information), then were brought to the University of Texas as live colonies (ants and garden). One queenright colony of the lower-attine ant *Mycetosoritis hartmanni* was collected in 2003 at Stengl Biological Station, Smithville, Bastrop Co., Texas. A queenright incipient leafcutter colony of *A. texana* was collected in Louisiana (2006; Leesburg, Vernon Parish, LA). In addition, queenless garden fragments with sufficient workers were collected cleanly from healthy gardens of three additional mature *A. texana* colonies throughout the range of this species in Texas (2007: Lake Houston Park, Harris Co.; Buffalo, Leon Co.; Graham, Young Co.). Additional collection details are summarized in the Supporting Information. All queenright ant colonies were maintained at the University of Texas at Austin using standard laboratory setups (Schultz 1993). For fungiculture, colonies received a mixed substrate of ground oat flakes, polenta, and dried oak catkins, with the exception of the leafcutter colonies that received leaf material from an ornamental pear (*Pryus calleryana*). At the time of microbial screening, each colony and garden appeared healthy and vigorous. Garden material was suspended and vortexed in agar-free broth of the respective media used in subsequent isolations (LBA, TSA, or chitin-agar; see below).

CULTURE-DEPENDENT ISOLATIONS BY DILUTION-TO-EXTINCTION

For bacterial isolation, we adapted Simu and Hagström's (2004) dilution-to-extinction method using 96-well Microtest plates (Becton Dickinson Labware, Falcon #351172, DIFCO, Becton Dickinson & Company, Sparks, MD). This method processes 12 parallel dilution series (columns 1–12) for each of seven samples (rows 1–7; using the 8th row as a control with sterile medium only). The first 96-well plate was prepared by pipetting 100 μl of original sample (suspension of either vortexed garden material or vortexed infrabuccal pellet; see sample preparation above) into 100 μl sterile medium per well (1:2 dilution), with 12 duplicates from the same original sample in the same row. Samples were then further diluted in 1:2 dilution steps by transferring 100 μl sample from one well of one 96-well plate into 100 μl sterile medium of the corresponding well of the next plate. Samples with garden material were diluted 11 times in 1:2 dilution steps by transfer between 11 successive plates; samples with pellet material were diluted seven times. Because of a higher bacterial load of garden material, more dilution steps were necessary to reach extinction for this type of sample. Two types of media were used for dilution-to-extinction isolation, LB medium and chitin medium. LB (Luria-Bertani) medium was supplemented with cycloheximide (50 mg/L) to suppress fungal growth. Liquid chitin medium was adapted from Cafaro and Currie (2005) by grinding chitin [betha-(1,4)-2-acetamido-2-deoxy-D-glucose; Acros Organics, Morris Plains, NJ] in a clean spice grinder, autoclaving 3 g of the ground chitin in 750 mL saline medium (0.575 g K_2HPO_4, 0.375 g $MgSO_4$, 0.275 g KH_2PO_4, 0.0075 g $FeSO_4$, 0.00075 g $MnCl_2.4H_2O$, 0.00075 g $ZnSO_4$, supplemented with 2 mg/mL of Nystatin and 50 mg/L of cycloheximide), and carefully pipetting the supernatant of the sterilized liquid medium

to avoid transfer of unsuspended chitin settling at the bottom of the flask. LB medium is a high-nutrient medium for the growth of diverse microbes, whereas the chitin medium is a minimum-nutrient medium that has been used to isolate *Pseudonocardia* associates of fungus-growing ants (Cafaro and Currie 2005). To test for the adequacy of our methods in isolating actinomycete bacteria, we also screened, as a positive control, one worker and one larva of the attine ant *Trachymyrmex arizonensis* (collected in August 2001 near Portal, Cochise County, Arizona). A *Trachymyrmex* species was chosen as a positive control because Cafaro and Currie (2005) and Currie et al. (2006) reported abundant actinomycete growth on *Trachymyrmex* workers. Plates were kept at room temperature (20–22°C) for at least two weeks (LB medium) or at least four weeks (chitin medium) before picking bacteria for sequencing (see below). For single pellets and garden fragments, respectively, 5–7 and 7–11 steps of 1:2 dilutions yielded growth in about 50% of the wells, and a random sample of the bacteria of these dilutions was used for sequencing. Pellets collected from *A. texana* queens from mating flights in 2005 ($n = 7$ pellets) and 2006 ($n = 14$), and seven fragments taken in 2005 from mature garden of a laboratory colony of *M. hartmanni*, were screened with the dilution-to-extinction method.

CULTURE-DEPENDENT ISOLATIONS BY PLATING

In addition to the above dilution-to-extinction isolations using liquid medium, we isolated bacteria by plating 100×, 1000×, and 10,000× dilutions on petri plates with one of three types of agar-solidified medium, LBA (Luria-Bertani agar, supplemented with 50 mg/L cycloheximide to suppress fungal growth), TSA (tryptic soy agar supplemented with 50 mg/L cycloheximide; Boureau et al. 2004), and chitin agar (1.5% agar in the chitin medium described above and adapted from Cafaro and Currie [2005]]. Dilutions were made with agar-free broth of the corresponding media. Gardens of six Panamanian attine ant species were screened in 2004 with these plating methods, one garden each for the lower attine ants *A. dentigerum*, *C. costatus*, *C. longiscapus*, and *C. muelleri*; from the higher attine *S. amabilis*; and from the leaf-cutter ants *A. colombica* and *A. coronatus* (because *M. hartmanni* colonies were not available in 2004, garden of this species was not screened with the plating method, but only with the dilution-to-extinction method developed in 2005). A 4-mm diameter garden fragment was excised from stabilized garden with sterilized forceps and transferred to a tube with 2 mL sterilized broth, vortexed for 5 min to disperse garden contents, then diluted for plating. Two such samples were taken from each garden and processed separately, one sample from younger garden near the top, one from older garden at the base and in the center. Plates were kept at room temperature (20–22°C) for at least two weeks (LBA and TSA) or at least four weeks (chitin agar) before picking bacteria for sequencing.

16S DNA SEQUENCING OF ISOLATES

Bacterial isolates were characterized by sequencing a segment of the 16S rDNA gene on an ABI PRISM 3100 16-channel capillary sequencer. For the isolates from 2004 and 2005, a portion of the 16S gene was amplified using the primer pair U519F and UA1406R (Baker et al. 2003). A minute amount of live bacterial isolate was transferred with a pipette tip to the PCR mix (1 μl 10× Buffer, 1 μl $MgCl_2$ 25 mM, 0.2 μl dNTP mix [2.5 mM of each nucleotide], 0.3 μl of each primer 10 mM, 0.02 μl Taq polymerase, ddH_2O to a total volume of 10 μl), heated for 10 min to 96°C to release DNA, which was then amplified (35 cycles of 1 min 95°C, 1 min 54°C, 2 min 72°C; then a 10-min extension at 72°C). Amplification yielded sequencing template of about 900 bp, and forward and reverse sequencing of this template gave about 500–800 bp overlap to compile a contig spanning the template sequence. For the isolates from 2006, a longer segment of the 16S gene was amplified and sequenced with the primer pair F27 and R1492 (Lane 1991) (1 μl 10× buffer, 1 μl $MgCl_2$ 25 mM, 0.8 μl dNTP mix [2.5 mM each nucleotide], 0.6 μl of each primer 10 mM, 0.03 μl Taq polymerase, 1 μl of BSA, 2 μl template, and ddH_20 to a total volume of 10 μl). In some cases, a contig of the roughly 1460 bp sequence could be compiled by end-sequencing (about 100–200 bp overlap); if this was not possible because of insufficient overlap, a portion of the forward sequence was analyzed with the help of the internal primer U519F, which was then complied with the information from the end-sequencing. All PCR products were cycle-sequenced with the ABI BigDye Terminator Kit (ver. 3.1) on an ABI PRISM 3100 automated sequencer.

PSEUDONOCARDIA-SPECIFIC 16S PRIMERS

Because our culture-dependent screens failed to reveal *Pseudonocardia* in queen-pellets of *A. texana* and in gardens of various attine ants (see Results), we developed a culture-independent screen to test for the adequacy of our culture-dependent methods. Culture-independent screens were also negative for *Atta* pellets and gardens, so we experimented with several extraction protocols to optimize DNA extraction from samples (pellet, garden fragment, or ant). In the most successful method (e.g., for *Trachymyrmex* and *Cyphomyrmex* ants as positive controls), samples were first frozen using liquid nitrogen and crushed using a plastic pestle. Samples were then placed in 100 μl of lysis buffer (0.1 M NaCl, 0.2 M sucrose, 0.1 M Tris [9.1 pH], 250 mL 0.05 M EDTA, 0.05% SDS] and heated in a block at 100°C for 15 min. Lysed samples were then immersed in 100 μl of a 20% Chelex buffer (Sigma-Aldrich, St. Louis, MO), incubated at room temperature for 15 min while vortexing, then heated again for 15 min to 100°C. A variation of this procedure was to boil the Chelex-suspended sample for 90 min. A more elaborate method involved an overnight incubation at room temperature in lysis buffer,

followed by CTAB extraction (Gerardo et al. 2004). All extracts were ultimately centrifuged at 12,000 rpm for 15 min, and 1 μl supernatant was used for PCR amplification.

Initially, we tried direct amplification with universal 16S rDNA primers (F27/R1492; Lane 1991), using for each PCR reaction 1μl of 10× PCR-buffer II (Applied Biosystems, Foster City, CA), 0.5 μl of 50 mM $MgCl_2$, 1 μl dNTP mix (2.5 mM of each nucleotide), 1 μl of each primer (at 10 mM), 0.03 μl of AmpliTaq polymerase (Applied Biosystems), and 1μl 100× BSA (New England BioLabs, Ipswich, MA). Template volume varied from 1.5 to 2.5 μl, and addition of PCR-grade water was adjusted to bring the final reaction volume to 10 μl. The following thermal profile was used with the 16S primers: 95°C for 45 sec, 60°C for 1 min, 72°C for 2 min, repeat for 35 cycles, followed by a final extension step of 72°C for 15 min.

Although amplification with universal 16S rDNA primers gave good sequences for extracts from the positive controls (*Trachymyrmex* and *Cyphomyrmex* ants; details below), amplification from *Atta* samples failed, or yielded unreadable sequences. We therefore optimized PCR conditions for the *Pseudonocardia*-specific 16S rDNA primer-pair AMP2/AMP3 (Morón et al. 1999). For the positive controls, the following recipe gave the strongest amplifications: 1 μl of PCR-buffer II (Applied Biosystems), 0.8 μl of 50 mM $MgCl_2$, 1 μl dNTP mix (2.5 mM of each nucleotide), 1 μl of each primer (at 10 mM), 0.07μl of AmpliTaq polymerase (Applied Biosystems), and 1 μl 100× BSA (New England BioLabs). Template volume varied from 1.0–2.0 μl, and addition of PCR-grade water was adjusted to bring the total reaction volume to 10 μl. Morón et al.'s (1999) recommended thermal profile was used with the AMP2/AMP3 pair: 93°C for 30 sec, 52.5°C for 40 sec, 72°C for 2 min, repeat for 40 cycles; followed by a final extension step of 72°C for 10 min. After sizing PCR products with agarose-gel electrophoresis, only products around the expected 670bp were sequenced. All PCR products were cycle-sequenced with the ABI BigDye Terminator Kit (version 3.1). Forward and reverse sequences were obtained using an Applied Biosystems ABI PRISM 3100 automated sequencer.

Because Currie et al. (1999b, 2006) had reported the presence of *Pseudonocardia* on *Atta* workers, but our culture-independent methods with both the universal and *Pseudonocardia*-specific primers failed to detect *Pseudonocardia* on *Atta,* we tried to improve our extraction procedure by extracting from specific body parts (in an effort to maximize bacterial DNA content while minimizing interference of PCR-inhibitors). In the positive controls (*Trachymyrmex* and *Cyphomyrmex* ants), the propleural plates were excised for DNA extraction. In *Atta*, both the propleural plates and the metapleural glands (reservoir and associated gland cells) were excised from minim and medium workers for separate DNA extraction. As a variation, the metapleural glands were left intact on the ant's body, but the glandular openings were increased to allow the secretion to ooze out into the extraction buffer. Even though the metapleural gland is thought to produce primarily antimicrobial secretions, we targeted this gland because it is hypertrophied in leafcutter ants (Hughes et al. 2008) and because Poulsen et al. (2003b) hypothesized that the secretions help sustain *Pseudonocardia* growth. All excision procedures of specific body parts were performed in addition to extracting entire worker ants.

Forty workers of different sizes (minims to medium workers) from four *Atta* species (one colony each of *A. cephalotes* and *A. sexdens*, two colonies each of *A. colombica* and *A. texana*) were independently extracted, then tested multiple times for the presence of *Pseudonocardia* under various PCR conditions. In addition, 38 garden fragments (2–4 mm diameter; collected with sterilized forceps from mature garden, as described above under garden collection) from the four *Atta* species were sampled repeatedly under various PCR conditions. PCR conditions varied in thermal profile (e.g., lower annealing temperature), template concentration (0.5–2 μl), $MgCl_2$ concentration (0.5–1.5 μl at 50 mM), BSA addition, and Taq-polymerase concentration (0.03–0.1 μl). Details on the exact combination of extraction variant and PCR variant can be obtained from the authors. A total of 166 PCR experiments (all unsuccessful) were completed on *Atta* (*A. cephalotes*: 21 worker, 21 garden; *colombica*: 23 worker, 8 garden; *sexdens*: 23 worker, 8 garden; *texana*: 26 worker, 27 garden, 9 pellet).

As positive controls, we used workers of various live *Trachymyrmex* and *Cyphomyrmex* ant colonies maintained in the Mueller laboratory (*T. arizonensis* and *T. desertorum* from Arizona; *T. septentrionalis*, *T. turrifex*, and *Cyphomyrmex wheeleri* from Texas; and *T. zeteki* from Parque Soberanía, Republic of Panamá).

SEQUENCE ALIGNMENT

Forward and reverse sequences were assembled into individual contigs using SeqMan II version 5.05 (DNASTAR), then compared via the BLAST to information available at GenBank in September 2007. We included sequence information from previous microbial screens of attine ants (Van Borm et al. 2002; Currie et al. 2003b; Cafaro and Currie 2005; Zhang et al. 2007). Closely related sequences (top 5–10 matches at GenBank) were included in preliminary neighbor-joining analyses. Because of the great number of taxa in these initial exploratory analyses (∼400 taxa), we eliminated duplicate sequences and near-identical sequences that did not add critical information for taxonomic placement of the bacteria sequenced in our screens. However, because of the significance of *Pseudonocardia* bacteria in the biology of attine ants, we retained most 16S-sequence information available at GenBank in September 2007 for *Pseudonocardia*, but excluded the following redundant sequences: duplicate sequences of the same described *Pseudonocardia* species, and duplicate sequences

from a large marine sediment survey from the Mariana Trench (AY974775-AY974796; retaining AY974793, which was almost sequence-identical to the remaining sequences of that survey). The exclusion of these taxa does not affect any of our conclusions, as these excluded sequences are nearly identical to other sequences included in the final datasets. After the completion of our phylogenetic analyses, additional 16S sequences from ant-associated *Pseudonocardia* were released at GenBank in early 2008 (Poulsen et al. 2007); these new sequences are closely allied to ant-associated sequences already present in our dataset, and rather than reanalyzing an ever-expanding dataset (new information on free-living *Pseudonocardia* is continually deposited at GenBank), we decided to proceed with publication of our results (inclusion of the new GenBank information only strengthens our conclusions). Our final dataset included 217 taxa in the Actinomycetales. The actinobacteria *Bifidobacterium bifidus* and *Alloscardovia omnicolens* were used as outgroups for rooting. Sequences were aligned in Clustal X (Thompson et al. 1999) and then adjusted manually in MacClade version 4.06 (Maddison and Maddison 2000) to correct obvious alignment errors, yielding an alignment of 1508 characters. Regions of uncertain alignment (163 characters total) were excluded in the phylogenetic analyses, leaving a matrix of 1345 characters (408 informative, 38 autapomorphic, and 899 invariable characters). The genus *Pseudonocardia* emerged as a monophyletic group in the global actinomycete analysis (Fig. 1), and a second alignment therefore could be compiled for the 86 taxa in this genus only (1482 total characters, 24 excluded characters of uncertain alignment; 209 informative, 47 autapomorphic, and 1202 invariable characters; Fig. 2), using as outgroup the six representatives in the genus *Amycolatopsis* (family *Pseudonocardiaceae*) that were already in the global actinomycete analysis (Fig. 1). Between global and specific analyses, the topology of the genus *Pseudonocardia* did not differ with respect to the key features stressed in our conclusions (differences involved a few minor rearrangements at the termina of near sequence-identical taxa), and we therefore show for *Pseudonocardia* only the phylogenetic reconstruction based on the specific analysis (Fig. 2). Alignments of both global and specific analyses are available from the corresponding author.

GENBANK DEPOSITION

Collection information, isolation methods, and culture-independent methods are summarized in Supporting Table S1. Collection and sequence information is also deposited at GenBank under accessions EU718276-EU718356.

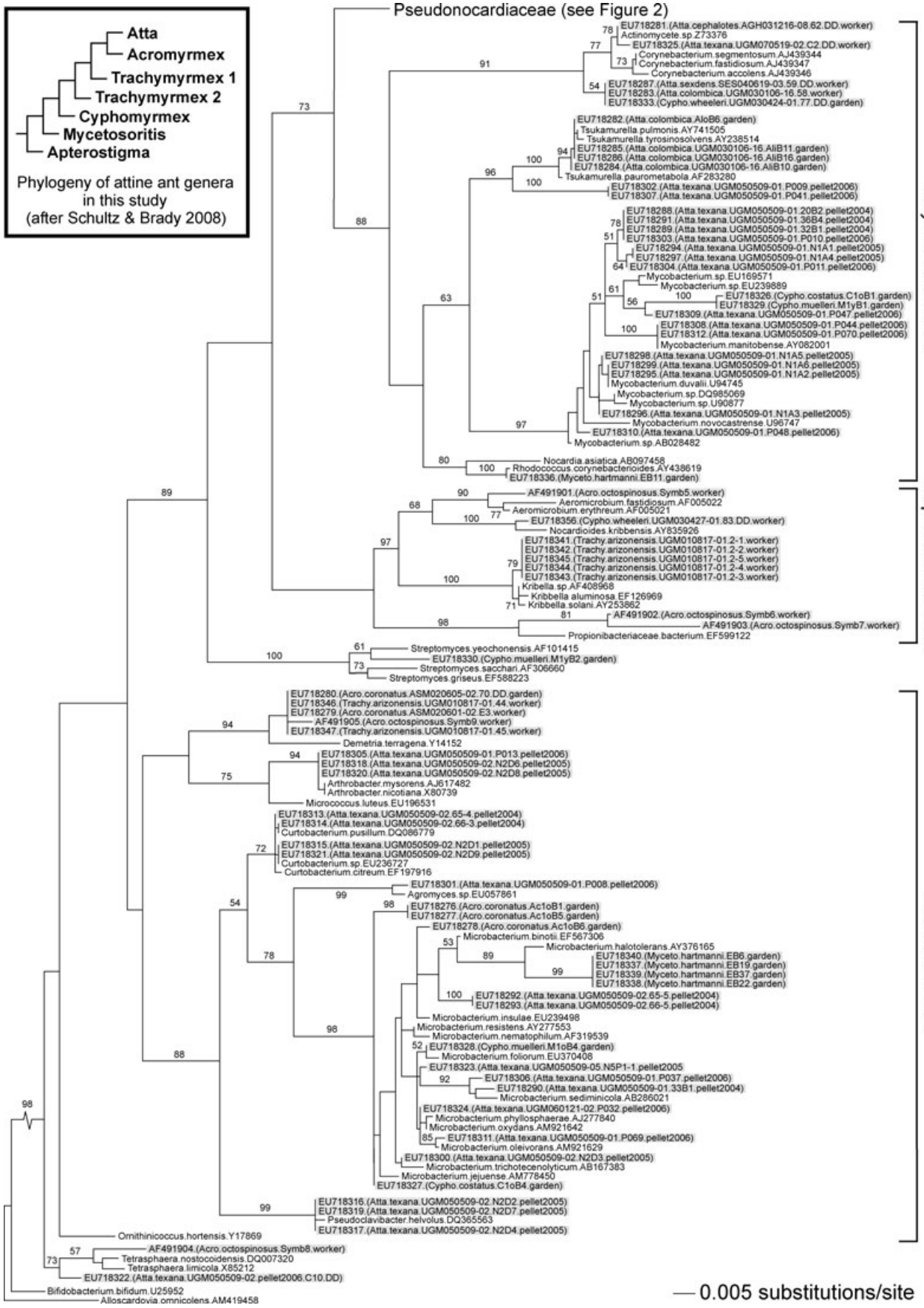
PHYLOGENETIC ANALYSES

Phylogenetic relationships were inferred under the maximum likelihood (ML) criterion using the General Time Reversible (GTR + I + G) model of nucleotide substitution (Garli ver. 0.951; Zwickl 2006; www.zo.utexas.edu/faculty/antisense/garli/Garli.html). The appropriate model of nucleotide substitution was selected using the Akaike Information Criterion (AIC) implemented in Modeltest 3.7 (Posada and Crandall 1998). As recommended in the Garli user manual, we performed 10 separate likelihood searches for each alignment to search likelihood space more thoroughly. In the global Actinomycetales analysis, these 10 searches recovered almost identical topologies, except in one reconstruction in which the monophyletic clade of the Propionibacterineae (representatives of *Aeromicrobium*, *Kribbella*, *Priopionibacterium*, and ant-associated bacteria affiliated with these genera) was the sister clade to the *Pseudonocardineae*; all other nine reconstructions inferred, as shown in Figure 1, the *Propionibacterinea* as the sister clade to [*Corynebacterineae* + *Pseudonocardineae*]). The log-likelihood value of the single anomalous tree was not significantly worse than the values of the other nine trees and therefore cannot be rejected. The uncertain position of the *Propionibacterineae* relative to the *Corynebacterineae* and the *Pseudonocardineae* does not change any of the conclusions emphasized below, which are based on the frequent isolation of ant-associated bacteria within the genera *Pseudonocardia*, *Mycobacterium*, and *Microbacterium*. No similar topological uncertainty emerged in the second, specific analysis on the genus *Pseudonocardia*. Branch support was calculated from 100 pseudorepetitions in a nonparametric, ML-bootstrap analysis. Figures 1 and 2 report the best topologies of the ML searches with the lowest log-likelihood scores.

Results

PHYLOGENETIC INFERENCES

Figures 1 and 2 present the phylogenetic relationships between ant-associated actinomycete bacteria (shaded) and closely related, nonattine bacteria (unshaded), integrating information from our culture-dependent and culture-independent screens with information available at GenBank in September 2007 (Supporting Table S1 lists the microbes identified by the various methods in our surveys). Even though our analyses did not include some prominent actinomycete lineages because no attine-associated representatives are known (e.g., family *Frankiaceae*, which include mutualists of plants), our phylogenetic reconstruction recovers basically the same topology found in previous analyses of 16S and protein-coding sequences (e.g., Gao and Gupta 2005, and references therein), with the *Micrococcineae* (including the family *Microbacteriaceae*) more basal among the Actinomycetales and the *Corynebacterineae* (including the families *Pseudonocardiaceae* and Mycobacteriaceae) more derived (Fig. 1). *Mycobacterium* and *Microbacterium* actinomycete bacteria were most frequently isolated (both 8% of all identified bacteria in our survey) in our culture-dependent screens of attine gardens and *A. texana* pellets.


Atta
Acromyrmex
Trachymyrmex 1
Trachymyrmex 2
Cyphomyrmex
Mycetosoritis
Apterostigma
Phylogeny of attine ant genera in this study (after Schultz & Brady 2008)
Pseudonocardiaceae (see Figure 2)
Corynebacterineae
Propionibacterineae
Micrococcineae
— 0.005 substitutions/site

CULTURE-INDEPENDENT SCREENS FOR *PSEUDONOCARDIA* FROM *ATTA* LEAFCUTTER ANTS

Although extracts from the positive controls (*Trachymyrmex* and *Cyphomyrmex* workers) consistently yielded sequenceable PCR products that could be identified as *Pseudonocardia* with the BLAST at GenBank (Fig. 2), all of the 166 PCR attempts of *Atta* extractions failed (*A. cephalotes*: 21 worker, 21 garden; *colombica*: 23 worker, 8 garden; *sexdens*: 23 worker, 8 garden; *texana*: 26 worker, 27 garden, 9 pellet). Because of the extensive variation in extraction and PCR conditions to facilitate amplification of *Pseudonocardia* from *Atta* extracts, the large number of PCR experiments (166 total), and amplifications from positive controls were successful, it is unlikely that our failure to detect *Pseudonocardia* in *Atta* is due to undersampling or inadequate molecular techniques.

Discussion

The most abundant attine ant-associated bacteria in our surveys fall into three genera, *Pseudonocardia* (5% in our survey; 21% when including also all published ant-associated *Pseudonocardia*), *Mycobacterium* and *Microbacterium* (both 8% in our survey; no ant-associated *Mycobacterium* or *Microbacterium* was known prior to our survey) (Figs. 1 and 2). The relative proportions of these most frequently isolated microbes reflect a combination of several factors, including isolation and sequencing biases (e.g., use of chitin media designed for isolation of *Pseudonocardia*; *Pseudonocardia*-specific primers) and the abundance of specific bacteria associated with attine ants. Consistent with previous reports (e.g., Currie et al. 2006), we found that it is comparatively easy to obtain *Pseudonocardia* sequences from the ant genera that served as positive controls (*Trachymyrmex* and *Cyphomyrmex*), presumably explaining also why much published information on ant-associated *Pseudonocardia* derives from these ant genera.

PSEUDONOCARDIA RECRUITMENT

Our most surprising finding was the close phylogenetic affinities between ant-associated and free-living *Pseudonocardia* (Fig. 2), which emerged when we integrated the information from ant-associated *Pseudonocardia* with the rich information on worldwide *Pseudonocardia* that have accumulated since Warwick et al.'s (1994) original 16S phylogenetic analysis of the genus *Pseudonocardia*. Surprisingly, many ant-associated *Pseudonocardia* are sequence-identical to free-living *Pseudonocardia*, and well-supported clades include closely related free-living and ant-associated *Pseudonocardia* that differ in only a few base pairs (Fig. 2). Moreover, sequence-identical free-living and ant-associated *Pseudonocardia* derive from geographically distant locations and disparate environments (e.g., marine sediment near China and Panamanian attine ants; industrial sludge from France and Argentinean attine ant). These phylogenetic affinities indicate that ant-associated *Pseudonocardia* are frequently recruited into ant-association from environmental sources, as hypothesized by Kost et al. (2007).

Kost et al.'s recruitment hypothesis is further supported by the observation that *Pseudonocardia* isolates from single ant species occur in diverse *Pseudonocardia* clades (e.g., *A. octospinosus* has actinomycetes from both the *alni*-group and the *compacta*-group of *Pseudonocardia*; *Trachymyrmex zeteki* has actinomycetes from the *alni*-group and the *thermophila*-group; Fig. 2). Most telling, perhaps, is the finding that three clades of *Pseudonocardia* known from *T. zeteki* ants (collected in central Panama from the same ant population) cluster by laboratory where they were characterized [Kansas (Cafaro and Currie 2005), Wisconsin (Zhang et al. 2007), and Texas (this study); see Fig. 2]. Moreover, the *Pseudoncardia* amplified from *T. zeteki* colonies kept at the University of Texas were most closely related to *Pseudonocardia* amplified from *C. wheeleri* colonies collected in Texas, which were kept in the same laboratory as the *T. zeteki* colonies from Panama (Fig. 2). This clustering by laboratory suggests that *Pseudonocardia* substitution may occur

Figure 1. Phylogenetic relationships of attine ant-associated actinomycete bacteria (shaded) and closely related free-living relatives (unshaded), inferred under the maximum-likelihood (ML) criterion from 16S sequence information. Attine-associated actinomycete bacteria fall mainly into the genera *Microbacterium*, *Mycobacterium*, and *Pseudonocardia*. The position of the family *Pseudonocardiaceae* is indicated as a stub at the top of the tree, and Figure 2 details the phylogenetic relationships within the genus *Pseudonocardia*. Support values are inferred from 100 ML bootstrap pseudoreplicates. For attine-associated actinomycete bacteria (shaded), each taxon label gives the GenBank accession, followed in parentheses by the ant species name from which a bacterium was sampled, the ant collection ID (e.g., UGM050509–01), the bacterium sample ID (e.g., P009, N2D2, or 66-5), and the material from which the bacterium was isolated (worker, garden, or pellet). Ant genera are abbreviated as: Acro, *Acromyrmex*; Aptero, *Apterostigma*; Cypho, *Cyphomyrmex*; Myceto, *Mycetosoritis*; Trachy, *Trachymyrmex*. Insert (top left). Phylogenetic relationships between the attine ant genera, showing only the genera included in this study. The genus *Trachymyrmex* is paraphyletic; the *Trachymyrmex* species studied here include representatives from *Trachymyrmex 1* (*arizonensis*, *desertorum*, *septentrionalis*) and *Trachymyrmex 2* (*cornetzi*, *turrifex*, *zeteki*).

Figure 2. Phylogenetic relationships of attine ant-associated (shaded) and free-living (unshaded) bacteria in the genera *Pseudonocardia* and *Amycolatopsis* (*Pseudonocardiaceae*). Attine-associated lineages are closely related to soil-dwelling or endophytic *Pseudonocardia* lineages, indicating frequent recruitment of free-living *Pseudonocardia* into the mutualism with ants. Support values are inferred from 100 ML bootstrap pseudoreplicates. For attine-associated bacteria, each taxon label gives the GenBank accession, followed in parentheses by the ant species name from which a bacterium was sampled, the ant collection ID (e.g., UGM010817-01), the bacterium sample ID (e.g., 57.DD), and the material from which the bacterium was obtained (worker, larva, or garden). The labels "Cafaro & Currie 2005," "Zhang et al. 2007," and "this study" highlight actinomycetes isolated from the same ant species *T. zeteki*, collected from the same population in Panama but processed in different labs (Kansas, Wisconsin, Texas, respectively), suggesting laboratory-specific biases. Ant genera are abbreviated as: Acro, *Acromyrmex*; Aptero, *Apterostigma*; Cypho, *Cyphomyrmex*; Myceto, *Mycetosoritis*; Trach, *Trachymyrmex*.

rapidly within the lifetime of laboratory ant nests, possibly mediated by different substrate fed in the different laboratories to the ants, by different nest setups, by different vectors that may ferry *Pseudonocardia* between laboratory nests, or other such sources of cross-contamination. In sum, the accumulated evidence appears to support Kost et al's view of ant-*Pseudonocardia* association that is as dynamic and as diffuse as the association between *Rhizobium* bacteria of root-nodulating plants, but not as specific as predicted previously for the ant–actinomycete symbiosis (Currie et al. 1999b; Currie 2001a; Poulsen and Currie 2006).

The identity between some ant-associated *Pseudonocardia* and soil/endophyte isolates (Fig. 2) implicates these environments as sources from which attine ants may acquire *Pseudonocardia*. The soil-dwelling habits of many attine ants expose them to a great diversity of microbes, including soil actinomycetes (Lazzarini et al. 2000; Basilio et al. 2003), particularly during the ants' tunneling activities. Some lower-attine species even incorporate soil particles into their gardens (U.G. Mueller, pers. obs.). Moreover the foraging and gardening behaviors of attine ants may be modulated by the presence of microbes in specific substrate (e.g., leaves of specific plant species, insect frass, decaying plant matter). Actinomycete recruitment and turnover therefore may be a function of many ant-specific factors, such as foraging behavior, substrate choice, and nesting preferences (soil vs. arboreal). For example, leaf fragments that have been processed by *Atta* and *Acromyrmex* leafcutter workers show small actinomycete loads, whereas unprocessed leaf material is thought to be free of actinomycetes (Mangone and Currie 2007). This could mean that leafcutter workers inoculate actinomycetes onto garden substrate (Mangone and Currie 2007), or that workers release endophytic *Pseudonocardia* from the leaves during substrate preparation, or both. The close phylogenetic relationship between ant-associated and endophytic *Pseudonocardia* (Fig. 2) supports this later hypothesis of actinomycetes release during leaf mastication (but does not rule out the first hypothesis of active inoculation). If endophytic *Pseudonocardia* are released from leaf tissue and integrated into the garden matrix, transfer from garden to ant integument may occur occasionally. Such substrate-mediated transfer of actinomycetes could even lead to spurious clade-to-clade correspondences between ant and actinomycete phylogenies, as different actinomycete lineages may inhabit live, fresh vegetation (substrate preferred by leafcutter ants) than inert, dried vegetable matter (substrate used by lower attine ants). Future work should test this hypothesis of actinomycete-release from plant substrate in carefully controlled experiments.

The exposed accumulations of the integumental microbes of attines suggests adaptive design as an open or semiopen system, premised on continuous pickup of novel microbes from environmental sources, rather than long-term association ensured by sequestration of a specific microbial population in a pouch or receptacle. Microbial substitution on the exposed integument is likely constrained by interbacterial competition once an actinomycete population is established on the integument (Poulsen et al. 2007), such that the inoculation with nest-specific microbes of newly eclosed workers immediately after eclosion ensures some continuity within attine nests (Poulsen et al. 2003a). However, the location of the actinomycete growth on exposed body structures, such as the venter, the legs, the gaster, the face (frontal lobes at the base of the antennae), and the propleural plate seem designed as a relatively uncontained system, as if regular import or substitution is adaptive. Control over the persistence of a particular microbe association likely exists in the bacteria-housing cuticular pits of *Cyphomyrmex* and possibly also the thoracic depressions (propleural foveae) that are partly shielded by hairs (Currie et al. 2006). However, control is compromised in the exposed microbial accumulations (venter, dorsum, legs, or gaster), particularly in those attine genera that have initial actinomycete colonization on minute, exposed integumental tubercles (e.g., *Acromyrmex*; Currie et al. 2006). Dynamic turnover, as envisioned by Kost et al. (2007), thus may be an inevitable property of the relatively open, unprotected design of the integumental microbial growth. More detailed analyses of the distribution and layerings of the various microbes across the integument of attine ants are needed to evaluate any adaptive value of the openness or semiopenness of the ant-bacteria association.

RARITY OR ABSENCE OF *PSEUDONOCARDIA* IN *ATTA* LEAFCUTTER ANTS

We were not able to confirm that *Pseudonocardia* is associated with ants in the leafcutter genus *Atta*, despite our multiple and varied attempts, including culture-independent methods targeting whole workers and specific body regions (propleural plate, metapleural gland) of four species of *Atta*, as well as culture-dependent and culture-independent methods targeting *Atta* gardens and the infrabuccal pellets carried by queens of *A. texana*. The four *Atta* species screened (*A. cephalotes*, *colombica*, *sexdens*, *texana*) represent three of the four main subgroupings recognized in the genus *Atta* (*Neoatta*, *Archeatta*, *Atta s.str*; Borgmeier 1959; Bacci et al., 2008). Because workers from multiple nests were tried repeatedly for these diverse species (at least 10 workers extracted per species; multiple PCR attempts under varying PCR conditions per extract), it seems unlikely that our failure to detect *Pseudonocardia* is due to undersampling.

Our difficulty in finding *Pseudonocardia* associated with *Atta* is also reflected by the fact that, to date, only four *Pseudonocardia* isolates have been reported from *Atta* (Zhang et al. 2007; Poulsen et al. 2007; Fig. 2), whereas five times that number have been reported from each of *Trachymyrmex* and *Acromyrmex* (Fig. 2). Moreover, Fernández-Marín et al. (2006b;) report colony-forming units (CFU) in the order of 10^6 when isolating

actinomycete bacteria from the integument of *Acromyrmex* workers, but almost none (<1 CFU) when isolating from *Atta*, suggesting that actinomycetes on *Atta* occur at the level of environmental contamination. Previous reports gave the impression that actinomycete bacteria can be obtained from *Atta* just as readily as from other attine genera (e.g., Currie et al. 1999b, 2006). Specifically, Currie et al. (1999b, p. 702) reported the "consistent association of the actinomycete with diverse attine ants," including three species of *Atta*, and that, in 112 attine colonies studied (including *Atta* and *Sericomyrmex* colonies) "in all cases, the actinomycete was concentrated on genus-specific areas of the ant integument that appear to be modified for the maintenance and growth" of the actinomycete. A later study (supporting online material of Currie et al. 2006, p. 4) corrected this initial report by cautioning that "two genera, *Sericomyrmex* and *Atta*, have no filamentous bacterium or morphological structures present on the external exoskeleton. However in vitro isolations from workers of both genera yielded filamentous bacteria indicating that mutualistic bacteria are present, although the location of the bacteria is unknown." The accumulated evidence now suggests that *Pseudonocardia* in *Atta* is comparatively rare or perhaps absent. The close phylogenetic affinities between the four reported *Pseudonocardia* from *Atta* and various *Pseudonocardia* from soil or plant material (Fig. 2; see discussion below) could even suggest that the four reported isolates from *Atta* represent accidental contaminants, as *Pseudonocardia* is likely found as contaminant on any soil-dwelling insect.

MYCOBACTERIUM AND *MICROBACTERIUM* IN ATTINE GARDENS

Even though our methods failed to detect *Pseudonocardia* associated with *Atta* ants, we were able to document the consistent presence of other actinomycete bacteria in gardens of *Atta* and other attine ant species, as well as in queen-pellets of *A. texana* (harvested in three successive years). The most prominent of these bacteria were in the genera *Mycobacterium* and *Microbacterium* (Fig. 1). These actinomycetes could play disease-suppressing or other unknown roles in gardens of attine ants, for example when they become incorporated via infrabuccal pellets in incipient gardens and then are copropagated with the cultivated fungus by workers (Mueller et al. 2005). However, as intriguing as this hypothesis on fungus-actinomycete copropagation may seem, a simpler explanation is that *Microbacterium* and *Mycobacterium* are prevalent in *Atta* pellets and attine gardens because the ants accidentally import these bacteria as they incorporate plant material into gardens and pellets. *Microbacterium* and *Mycobacterium* are abundant components of bacterial communities in soil and leaf material (as is *Pseudonocardia*; Goodfellow and Williams 1983; McCarthy 1987; Conn and Franco 2004; Rosenblueth and Martínez-Romero 2006; El-Tarabily and Sivasithamparam 2006), and the prevalence of these actinomycetes in attine gardens is perhaps not surprising. Future work should assess whether the *Microbacterium* and *Mycobacterium* that are transmitted via infrabuccal pellets could perhaps serve analogous functions that *Pseudonocardia* appears to serve on the integument of attine ants, or alternatively, whether these garden-associated actinomycetes are unavoidable contaminants of minor or no importance to attine ants. As parallel cases, Santos et al. (2004) had argued that *Burkholderia* bacteria in *Atta* gardens may serve disease-suppressing functions inhibiting *Escovopsis* and entomopathogenic fungi of attine ants, and Cardoza et al. (2006) showed that the actinomycete *Micrococcus luteus* (a relative of *Microbacterium*) in secretions of fungus-growing beetles can suppress several problem fungi of the beetles.

Although several attine ant-associated actinomycetes in our survey are possibly garden contaminants (e.g., *Curtobacterium*-like and *Pseudoclavibacter*-like isolates; Fig. 1), several derived clades of ant-associated actinomycetes seem of interest, such as the derived *Microbacterium*-types isolated from the lower attine *M. hartmanni*, for which no closely related *Microbacterium* are known to date. A second group of interest could be the *Demetria*-like actinomycetes (Fig. 1), which were obtained from two *Acromyrmex* and one *Trachymyrmex* nest in our survey, and which had been obtained from *Acromymrex* workers in a previous study (Van Borm et al. 2002). A third group includes the *Kribella*-like isolates from *T. arizonensis*, which we obtained from the single positive control in the dilution-to-extinction experiment at greater frequency than *Pseudonocardia*. Such derived phylogenetic position or consistent association could indicate unique roles in the attine-microbe symbiosis.

PSEUDONOCARDIA SYSTEMATICS

Lee et al. (2000) recognized three groups in the genus *Pseudonocardia*, which conform to the groupings found in our study, except that the larger number of taxa now allows further subdivision. Based on the representative first described as a *Pseudonocardia* in each group, we have provisionally labeled these main groups in Figure 2 as the *thermophila* group, *halophobica* group, *compacta* group, and *alni* group (the last two subgroups are collapsed into a single group by Lee et al. 2000). The three main clades of ant-associated *Pseudonocardia* recognized by Zhang et al. (2007; called Clades I-III in that study) match respectively onto the *alni*, *compacta*, and *thermophila* groups shown in Figure 2 (except that the only nonattine-associated *Pseudonocardia* included in Zhang et al. 2007 were *P. halophobica* and *P. saturnea*; i.e., the phylogenetic relationships recovered in Zhang et al. were essentially the same as in Figure 2, but with virtually all of the free-living representatives pruned). Ant-associated *Pseudonocardia* are known from the *alni*, *compacta*, and *thermophila* groups, but so far not for the *halophobica* group. All four groups contain free-living *Pseudonocardia* from soil and marine sediment,

but the *alni* and *compacta* groups also contain *Pseudonocardia* symbiotic with marine sponges, plant roots, and live leaves (endophytic *Pseudonocardia*), as well as the great majority of ant-associated *Pseudonocardia*. In addition, the *Pseudonocardia* of *T. septentrionalis* appear to belong to the *alni* group (Mikheyev et al., 2008; the study used sequence information from the EF-Tu gene, rather than the 16S gene in the present study), and a *Pseudonocardia* belonging to the *compacta* group was recently found in a termite gut (Kurtböke and French 2007; the sequencing information of this study had not been deposited at GenBank in September 2007, therefore postdating our analyses). This could suggest ubiquitous symbiotic affiliations between diverse multicellular organisms and *alni*-type or *compacta*-type *Pseudonocardia*. A more comprehensive analysis of symbiotic and free-living lineages is needed to evaluate the symbiotic predispositions of specific lineages within the genus *Pseudonocardia*.

A fifth group is comprised of three *Trachymyrmex*-associated *Pseudonocardia* that appears as a basal clade in Figure 2. This well-supported group was reported in Cafaro and Currie's (2005) first sequencing study of attine actinomycetes as a possible *Trachymyrmex*-specific clade. No free-living relatives are yet known for these *Trachymyrmex*-symbionts; they appear to be unusual types, as all *Trachymyrmex*-associated *Pseudonocardia* reported subsequently belong to other groups (Zhang et al. 2007; Poulsen et al. 2007; our study). The position of this clade at the base of the genus *Pseudonocardia* is not well supported in our analysis, because likelihood reconstructions with slightly less support place this clade into or near the *halophobica* group. Uncertainty over the exact placement of this monophyletic clade does not alter any of the conclusions of our study, but it is the likely cause of the low bootstrap support values of the basal branching patterns in the genus *Pseudonocardia*. Apart from this unusual *Trachymyrmex*-associated clade, no well-defined clade of *Pseudonocardia* is associated with a specific ant clade, suggesting either frequent recruitment from free-living *Pseudonocardia* populations (Cafaro and Currie 2005; Kost et al. 2007), frequent horizontal transfer of *Pseudonocardia* between attine lineages (Cafaro and Currie 2005; Poulsen et al. 2005, Poulsen and Currie 2006), or both. Some *Trachymyrmex* workers even have actinomycetes in the closely related genus *Amycolatopsis* (also in the family *Pseudonocardiaceae*) (Fig. 2), adding to the number of "aberrant" associations that as a whole implicate recruitment from environmental sources. The association with bacteria in the genus *Amycolatopsis* is intriguing because members of this genus are sources of well-known pharmaceuticals, such as rifampicin and vancomycin.

A REASSESSMENT OF THE ATTINE ANT-ACTINOMYCETE SYMBIOSIS

Coevolution, like adaptation, is an onerous concept (Williams 1966). It should be invoked only when there is sufficient evidence for reciprocal selection (Janzen 1980; Futuyma and Slatkin 1983). Reciprocal selection is most readily documented in cases of specific (one-to-one) coevolution with unambiguous clade-to-clade correspondences, but it is more difficult to establish in cases of diffuse coevolution, particularly if one of the interactants can live an independent, free-living existence. The accumulated evidence now identifies the attine ant–actinomycete symbiosis as this latter type, with close phylogenetic ties between ant-associated and free-living *Pseudonocardia*, and no clear clade-to-clade correspondences between ant and bacterial partners. The clustering of *Pseudonocardia* lineages from the same ant species (*T. zeteki*) by study in which they were identified (Fig. 2; see clades labeled "Cafaro&Currie," "Zhang et al.," and "this study") even calls for a reevaluation of the microbial methods, as the clustering by laboratory suggests either laboratory-specific isolation and identification biases, or rapid replacement by laboratory-specific actinomycete strains once an ant colony is removed from its natural environment.

The accumulated evidence raises three concerns:

(1) Studies based on actinomycete isolations from attine ants without exact identification can no longer assume that unidentified actinomycete isolates are *Pseudonocardia* or necessarily mutualistic (Little et al. 2006; Mangone and Currie 2007), particularly in *Atta* leafcutter ants, which may form no functional associations with *Pseudonocardia*. Future studies will need to characterize more carefully the relative abundances and identities of actinomycetes that can be isolated from attine ants.

(2) Arguments based on the assumption of specific coevolution between attine ants and actinomycetes (Currie et al. 1999b) need to be revised, particularly speculations invoking complex Red-Queen arms-race scenarios of antibiotic-defense evolution without effective resistance evolution in *Escovopsis* (Currie 2001a; Salles et al. 2006; Poulsen and Currie 2006; Kumar et al. 2006).

(3) Popularizations of the attine ant-actinomycete symbiosis (e.g., Holzmann 2006; Kumar et al. 2006; Diamond 2006; Youngstaedt 2008) as a model for human antibiotic strategies that could be invulnerable to resistance evolution now appear to have been misguided by the earlier speculations of specific coevolution within a long-lasting arms race between *Pseudonocardia* and *Escovopsis*. The secret of attine actinomycete defenses may lie less in the harnessing of Red-Queen disease processes to prevent resistance evolution, but more in the frequent recruitment of diverse actinomycetes from environmental sources (sensu Kost et al. 2007), perhaps paralleling the continuous hunt for novel microbial activities by the pharmaceutical industry.

The integumental modifications of the ants to nourish and house cuticular microbes (Currie et al. 2006) and the associated metabolic cost to the ants in maintaining the microbial growth (Poulsen et al. 2002) provide evidence for selection on ants evolving in association with microbes. Evidence for the reverse—selection on the microbes evolving in a key interaction with the ants—seems harder to come by, particularly because the evolutionary fate of the bacteria may be shaped more by their free-living existence, rather than ant-associated existence. At present, only a single study of a single *Pseudonocardia* strain supports evolutionary modification on *Pseudonocardia*, namely the narrow antimicrobial specificity against *Escovopsis* reported by Currie et al. (1999b; i.e., absence of general antimicrobial activity). Given the frequent recruitment of *Pseudonocardia* from environmental sources (Fig. 2), however, integumental *Pseudonocardia* with at least some antifungal activities should exist, paralleling essentially the diverse antibacterial activities reported recently for the ant-associated *Pseudonocardia* (Poulsen et al. 2007). It is possible that only those *Pseudonocardia* strains are recruited that possess narrow antifungal properties effective only against *Escovopsis*, but such a scenario would require complicated mechanisms of acquisition (i.e., the exclusion of *Pseudonocardia* with broad antifungal activities). In addition, it is unclear how such narrow, *Escovopsis*-targeting antibiotic activities would be maintained in free-living *Pseudonocardia* populations, as *Escovopsis* does not appear to exist independent of attine nests (Currie et al. 1999a, 2003c). Alternatively, perhaps newly acquired actinomycetes rapidly loose generalized antifungal activities once associated with ants while simultaneously evolving *Escovopsis*-specific antifungal activity; this scenario assumes several parallel, complex modifications of actinomycetes once they become associated with ants, which must progress sufficiently rapid before strains are replaced within the apparently natural turnover of actinomycete communities on the attine integument. Clearly, more work is needed to assess whether the reported, narrow antifungal activity of *Pseudonocardia* is an anomaly, or representative for the ant-associated *Pseudonocardia* at large. Future work on attine actinomycetes (*Pseudonocardia*, *Mycobacterium*, *Microbacterium*, etc.) should reassess activities against microbes other than *Escovopsis*, essentially testing for multifarious purposes of beneficial microbes on the ants and in the gardens (e.g., as defenses against entomopathogens), as first suggested by Santos et al. (2004).

Conclusion

Nearly a decade after the first report of the attine ant-actinomycete symbiosis, our phylogenetic analyses prompt us to question the current belief of specific coevolution between attine ants and actinomycete bacteria, following the lead of Kost et al.'s (2007) recent suggestion of a dynamic and open ant-actinomycete system that is built on regular bacterial substitution and recruitment from free-living populations. Such conceptual revision does not make the attine-actinomycete symbiosis less interesting. In fact, comparisons with other diffusely evolving, open systems, such as rhizobium-plant mutualisms, polyp-algal mutualisms (corals), or fungal-algal mutualisms (lichens), may provide clues for critical future investigations on attine ants and their diverse actinomycete microbes. A comparison with rhizobium–plant mutualisms is particularly instructive, as the key evidence for the evolutionary modification of the rhizobial bacteria rests on the existence of nodulating genes without which a bacterium cannot mediate the entry into a mutualistic association with a host plant (Sawada et al. 2003; Sachs and Simms 2008; Kiers and Denison 2008). Testing for signatures of analogous genetic and cellular modifications in both free-living and ant-associated *Pseudonocardia* is a possible route to establish reciprocal coevolutionary modifications in the attine ant-*Pseudonocardia* symbiosis.

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Supporting Information

The following supporting information is available for this article:

Table S1. Sample information and isolation methods of the actinomycete bacteria sequenced for phylogenetic analyses. The information is also available at GenBank under the respective accession listed in the first column.

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