

Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in Fungus-Growing Ants

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Attine ants engage in a quadripartite symbiosis with fungi they cultivate for food, specialized garden parasites, and parasite-inhibiting bacteria. Molecular phylogenetic evidence supports an ancient host-pathogen association between the ant-cultivar mutualism and the garden parasite. Here we show that ants rear the antibiotic-producing bacteria in elaborate cuticular crypts, supported by unique exocrine glands, and that these structures have been highly modified across the ants' evolutionary history. This specialized structural evolution, together with the absence of these bacteria and modifications in other ant genera that do not grow fungus, indicate that the bacteria have an ancient and coevolved association with the ants, their fungal cultivar, and the garden parasite.

Attine ants are a New World tribe having obligate associations with fungi that they cultivate for food. The ants' fungal gardens are host to specialized and virulent parasitic microfungi in the genus *Escovopsis* (Ascomycota, Hypocreales) (1–3). Infected colonies experience a significant reduction in garden growth rate and production of new workers, and under some conditions *Escovopsis* can completely overgrow the fungus garden (1, 2). The symbiotic association between attine ants,

their fungal cultivars, and the specialized garden parasite *Escovopsis* originated about 50 to 65 million years ago and has subsequently been shaped by millions of years of coevolution (4–6). The ant-cultivar-*Escovopsis* host-pathogen coevolution has resulted in ancient evolutionary congruence: Specific groups of attine ants are specialized on specific groups of cultivated fungi, and these fungi are host to specific groups of *Escovopsis* parasites (4). In addition, even at the finer phylogenetic level, there is para-

site specialization on fungal cultivar genotypes (7).

To help defend their cultivar from the garden parasite, attine ants have a mutualistic association with filamentous bacteria that produce antibiotics with potent antagonistic properties against *Escovopsis* (3, 8, 9). The filamentous bacteria are in the genus *Pseudonocardia* (10); belonging in the order Actinomycetales, a group well known for its ability to produce antibiotics (11). *Pseudonocardia* bacteria are associated with all attine-ant species examined, and occur on specific locations on the cuticle of a given ant species. The bacterium is carried by gynes (female reproductive ants) on their mating flights and is thereby transmitted from parent to offspring colonies (8). Individual ant nests are associated with a single strain of *Pseudonocardia*, but genetically distinct strains

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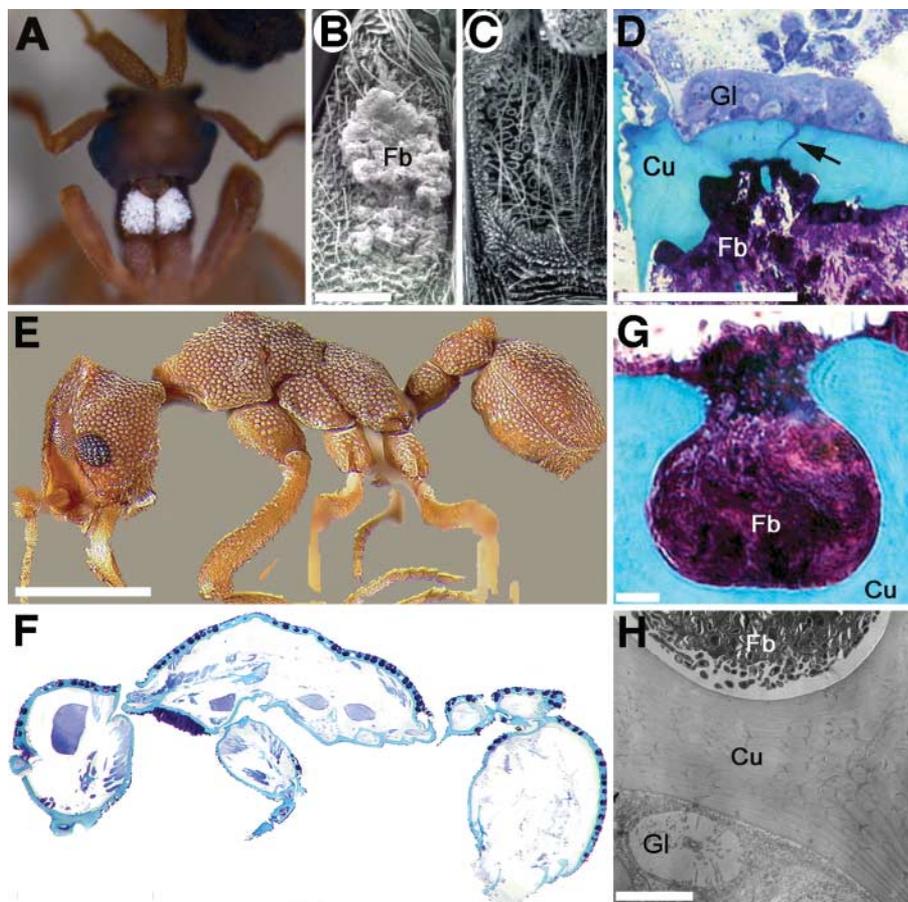
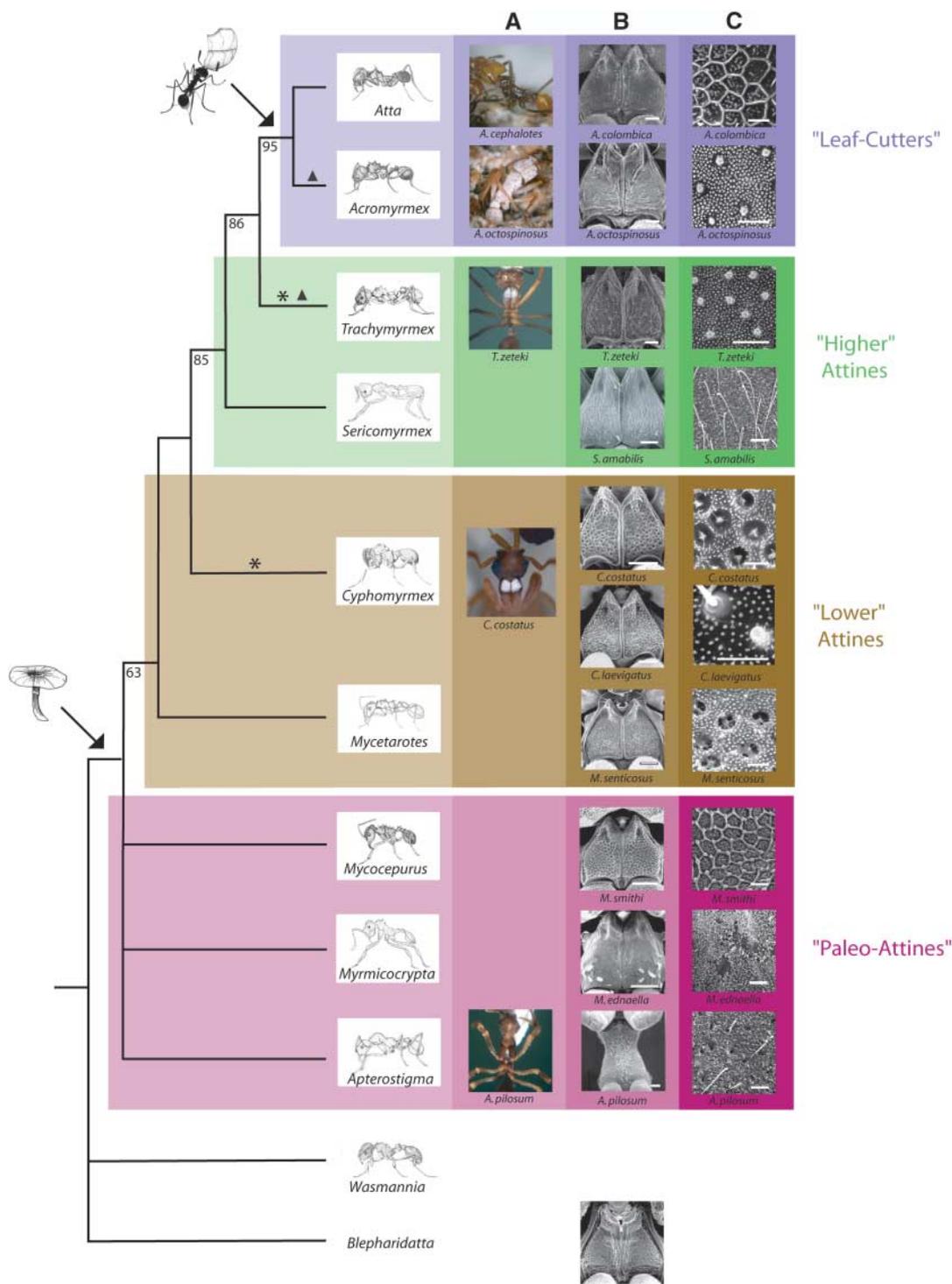


Fig. 1. (A) Photograph of *Cyphomyrmex costatus* showing the bacteria on the propleural plates. SEM of the plates in *C. muelleri*: The left plate is covered with bacteria (B), whereas they have been removed from the right plate, revealing the underlying fovea (C). (D) Light micrograph of a semithin cross section through the propleural plate of *C. longiscapus* showing the gland (Gl) and duct cells (black arrow) associated with the fovea and the bacterium (Fb) on the plate (Cu for cuticle). (E) Photograph of *C. longiscapus*, illustrating foveae openings covering most of the cuticle. (F) Sagittal semithin section through a *C. longiscapus* worker, illustrating foveae outlining nearly the entire body of the ant. (G) Light micrograph of a single fovea within the cuticle (Cu) illustrating the abundance of mutualistic bacteria (Fb) within the crypt. (H) TEM of the lower section of a fovea showing a single glandular cell (Gl) and bacteria (Fb) within the crypt. Scale bars: 50 μm (A to C), 5 μm (E and G), and 0.5 μm (F). [Photograph in (A) by A. Little]

Fig. 2. Genus level phylogeny of fungus-growing ants [adapted from (15, 16)] illustrating the location and modifications of the exoskeleton for maintaining the mutualistic bacteria. The origin of fungus growing by attine ants and the leaf-cutters is represented by the Lepiotaceous mushroom and the worker carrying a leaf fragment, respectively. Major groups of attine ants are depicted by colored boxes, illustrating the phylogenetically basal genera in the "paleo-attines" (red), the "lower" attine genera (brown), the "higher" attines (green), and the leaf-cutters (blue). (Column A) Photographs illustrate the location of the bacterium under the forelegs in the paleo-attines, on the propleural plates in the "lower" and "higher" attines, the presence all over the integument in the genus *Acromyrmex*, and absence on the cuticle in *Atta*. (Column B) SEM micrographs of the location of the bacterium, under the forelegs in *Apterostigma* and on the propleural plates in other groups. (Column C) SEM micrograph close-ups for the structures presented in (B), showing the specific structural modifications for different groups of fungus-growing ants. Presence of foveae (star) and tubercles (triangle) all over the body in some species within a genus are indicated by the corresponding symbol above the branch on the phylogeny. Scale bars: 0.5 mm (B), 10 μ m (C). [The line drawings of *Wasmannia auropunctata*, *Cyphomyrmex rimosus*, *Trachymyrmex septentrionalis*, *Acromyrmex versicolor*, and *Atta texana* were made by Smith (20); those of *Myrmicocrypta ednaella* and *Sericomyrmex amabilis* were made by Weber (21); and those of *Apterostigma pilosum*, *Mycocarpus smithi*, and *Mycetarotes* sp. were made by A. Little. Photographs of *Acromyrmex octospinosus* and *Cyphomyrmex costatus* in (A) were taken by A. Little.]



and/or species of bacteria can occur within populations of the same species and between species of ants (12). The diversity and mode of transmission predict congruence between the ant and bacteria phylogenies; however, the complete evolutionary history of ant-associated *Pseudonocardia* still remains to be determined. Here we examine the presence and evolution of specific cuticular structures on attine ants to

house and maintain the parasite-inhibiting bacteria (8).

To investigate this, we first examined ant species in the genus *Cyphomyrmex* because of the conspicuous white "bloom" of bacterium present on the propleural plates (Fig. 1A). Scanning electron microscopy (SEM) of *Cyphomyrmex longiscapus* and *C. muelleri* workers with the filamentous bacterium removed

revealed the presence of a previously unnoticed large crescent-shaped cavity (fovea) on each propleural plate (13) (Fig. 1, B and C; fig. S1A). The foveae are porous and occupy a significant proportion of the surface area of the propleural plates; the filamentous bacteria grow directly within these crypts (Fig. 1C).

Our investigations further revealed, in the semithin sections of the propleural plates in *C.*

longiscapus, the presence of a previously unknown exocrine gland located on the inner surface of the cuticle, just below the foveae. The gland consists of bicellular units, each formed by a gland cell and duct cell (14). The duct cells cross the cuticle and open within the foveae where the bacteria are cultured (Fig. 1D; fig. S1, A to C).

In addition to foveae occurring on the propleural plates, *C. longiscapus*, *C. muelleri*, and *C. costatus* ants also have bacteria-filled foveae covering most of the surface of worker exoskeletons, including the head, thorax, abdomen, and legs (Fig. 1, E to G). These crypts have small openings to the external surface of the ant, with minute microtrichia (hair-like cuticular projections) that appear to shield the opening of the crypt (fig. S1D). At the bottom of each fovea is a porous tubercle (integumental protrusion) (fig. S1E), connected via a duct cell to the corresponding gland cell directly beneath the crypt (Fig. 1H).

The locality of bacteria on the cuticle varies across fungus-growing ant species (Fig. 2, column A). Examination of specialized structures for bacterial maintenance across the phylogenetic diversity of attine ants revealed several broad evolutionary patterns (Fig. 2). Ant genera closely related to attine ants, *Wasmannia* and *Blepharidatta* (15, 16), do not have filamentous bacteria, foveae, or tubercles (Fig. 2). In the most phylogenetically basal attine ants (paleoattines), such as the genus *Apterostigma*, the filamentous bacterium occurs on the mesopleura (under the forelegs), where it grows directly on the cuticle over the pores of duct cells connected to the corresponding gland cells (Fig. 2, fig. S1F). In most species of “lower” attine ants, mutualistic bacteria occur on the propleural plates (e.g., *Cyphomyrmex costatus*, in Fig. 2), in which the bacterium grows on tubercles within foveae. Similarly, the bacteria are also concentrated on the propleural plates in the “higher” attine genus *Trachymyrmex* and the leaf-cutter genus *Acromyrmex*, although in these two genera the bacteria grow on gland cell-associated tubercles directly on the exoskeleton rather than in foveae (Fig. 2).

Several species of plants and animals engaged in mutualistic associations with microbes have evolved structures to house their symbionts. For example, root nodules in legumes house *Rhizobium*, squid light organs are filled with bioluminescent bacteria, aphids have modified bacteriocytes that form organlike structures to rear *Buchnera*, and some beetles and woodwasps have specialized structures (known as mycangia) to house mutualistic fungi (17–19). Our findings indicate that the exoskeleton of attine ants is modified to house mutualistic bacteria, apparently supporting their growth through glandular secretions. In addition, our phylogenetic examination of the structures across the fungus-growing ant tribe revealed that, like the cultivar and garden parasite, the

mutualistic *Pseudonocardia* bacteria was apparently present at the earliest stages of fungus cultivation by ants. This is supported by the presence of the bacteria and bacteria-associated glands and duct cells in the most phylogenetically basal genera (e.g., *Apterostigma*), in contrast to their absence in closely related ants that do not cultivate fungus gardens (*Blepharidatta* and *Wasmannia*).

The apparently early evolutionary origin of the bacteria within the fungus-growing ant symbiosis, in combination with bioassay results confirming that filamentous bacteria isolated from across the phylogenetic diversity of attine ants are effective at inhibiting their corresponding garden parasites (8, 10, 13), indicate that the bacteria have provided an efficient defense against *Escovopsis* for millions of years. This raises the question of how the antibiotics have remained effective without rampant evolution of resistance in the parasite over the long evolutionary history of this symbiosis.

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Supporting Online Material

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Materials and Methods
SOM Text
Figs. S1 and S2
References

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A Clonogenic Bone Marrow Progenitor Specific for Macrophages and Dendritic Cells

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Macrophages and dendritic cells (DCs) are crucial for immune and inflammatory responses and belong to a network of cells that has been termed the mononuclear phagocyte system (MPS). However, the origin and lineage of these cells remain poorly understood. Here, we describe the isolation and clonal analysis of a mouse bone marrow progenitor that is specific for monocytes, several macrophage subsets, and resident spleen DCs *in vivo*. It was also possible to recapitulate this differentiation *in vitro* by using treatment with the cytokines macrophage colony-stimulating factor and granulocyte-macrophage colony-stimulating factor. Thus, macrophages and DCs appear to renew from a common progenitor, providing a cellular and molecular basis for the concept of the MPS.

Macrophages (MΦs) and dendritic cells (DCs) are involved in the scavenging of dying cells, pathogens, and molecules through phagocytosis and endocytosis

and the use of pattern recognition receptors (1). As a result, both cell types make a vital contribution to immunity and inflammatory responses to pathogenic microorganisms (2).



Supporting Online Material for
Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in
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Materials and Methods
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Figs. S1 and S2
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Science Supporting Online Material

Materials and Methods

SOM Text: Results and Discussion

The role of cuticular and glandular structures in bacterial maintenance

Coevolutionary patterns in the structures supporting the bacteria

Bioassays

References

Figs. S1 and S2

Materials and Methods

Attine collection. Fungus-growing ants representing the phylogenetic diversity of the tribe were collected in Panama (canal zone), Ecuador (Tiputini, La Selva station), and Argentina (Misiones). Additional species of attine ants were obtained from the National Museum of Natural History, Smithsonian Institute, where all voucher specimens have been deposited. The material used in the investigation of the cuticular structures across the attine phylogeny presented in Fig. 2 were from the following ant species:

Apterostigma auriculatum, *A. dentigerum*, *A. pilosum*, *Mycocepurus smithi*, *Mycetophylax conformis*, *My. emeryi*, *Mycetarotes parallelus*, *Myc. senticosus*, *Mycetosoritis hartmani*, *Cyphomyrmex cornutus*, *C. costatus*, *C. faunulus*, *C. laevigatus*, *C. longiscapus*, *C. minutus*, *C. morshi*, *C. muelleri*, *C. rimosus*, *C. salvini*, *Sericomyrmex amabilis*, *Trachymyrmex cornetzi*, *T. zeteki*, *Acromyrmex balzani*, *Ac. echinator*, *Ac. insinuator*, *Ac. lundi*, *Ac. octospinosus*, *Atta cephalotes*, *At. colombica*, and *At. sexdens*.

Outgroups chosen for this investigation were *Blepharidatta* sp. and *Wasmannia auropunctata*.

Scanning electron microscopy (SEM). The presence of morphological structures associated with the filamentous bacteria in attine ants was examined using a scanning electron microscope (SEM, Philips 515). Specimens examined with the bacterium present were fixed in 2% glutaraldehyde buffer for 1 hour, dehydrated through a graded series of ethanol, and subsequently critically point dried. Workers examined for structures associated with the bacterium were cleaned using a 10% bleach solution for 1 hour.

Sectioning and transmission electron microscopy (TEM). Ants for sectioning and transmission electron microscopy were fixed in cold 2% glutaraldehyde in Na-cacodylate buffer. Postfixation was done in 2% osmium tetroxide and specimens were subsequently dehydrated in a graded acetone series. Specimens were embedded in Araldite and sectioned with a Reichert Ultracut E microtome. Semithin 1- μ m sections for light microscopy were stained with methylene blue and thionin. Double-stained 70-nm thin sections were examined in a Zeiss EM900 electron microscope.

Bioassay challenges between *Escovopsis* and the filamentous bacteria. To confirm the parasite-inhibiting role of the attine-ant associated actinomycetous bacteria across the diversity of the symbiosis, we tested whether actinomycete strains isolated from phylogenetically diverse attine ants inhibit their corresponding *Escovopsis*. More specifically, we performed bioassay challenges between strains of the actinomycetes and of *Escovopsis* isolated from colonies of each of the following ant genera: *Apterostigma*, *Cyphomyrmex*, *Trachymyrmex*, *Acromyrmex*, and *Atta*. Challenges were performed in Petri dishes (10 cm in diameter) on YMEA (0.4% yeast extract, 1% malt extract, 0.4% dextrose) (S1). Each bacterial isolate was challenged with a strain of *Escovopsis* that was isolated from the garden of the corresponding fungus-growing ant genus, and three replicates were performed for each pairing, except for *Cyphomyrmex* where only two were available. The actinomycete was inoculated in the center of the Petri dish and when having reached a diameter of \sim 1.5 cm, an *Escovopsis* inoculum was placed near the edge of the plate (S1). Inhibition of *Escovopsis* was scored as a reduction in the growth rate of *Escovopsis* and the formation of a zone of inhibition, which was measured after 15 days (cf. S1, S2).

SOM Text: Results and Discussion

The role of cuticular and glandular structures in bacterial maintenance. Multiple lines of evidence indicate that the glands associated with the tubercles and foveae produce nutrients that support the growth of the mutualistic bacteria. First, the bacteria grow on the ants' cuticle and are thus isolated from other possible nutrient sources. Second, removal of bacteria from the integument revealed no sign of damage to or penetration of the exoskeleton (Fig. 1), suggesting that the bacteria are not using the cuticle as a food source. Third, early growth of the bacteria on the integuments of

Cyphomyrmex costatus and *Acromyrmex octospinosus* is localized to the porous tubercles (fig. S2, A–H), which are connected to gland cells by cuticular duct cells. Fourth, except in the most basal genera (e.g., *Apterostigma*), species with filamentous bacteria on the cuticle have tubercles or foveae. Fifth, experimental infection of nests with the garden parasite *Escovopsis* results in a higher abundance of bacteria on garden tending works, suggesting that individual workers are able to control the growth of the bacterium, up-regulating the abundance of filamentous bacteria in the presence of *Escovopsis* infection (S3).

Coevolutionary patterns in the structures supporting the bacteria. The locality of bacteria on the cuticle varies across fungus-growing ant species (Fig. 2, column A). Examination of specialized structures for bacterial maintenance across the phylogenetic diversity of attine ants revealed several broad evolutionary patterns (Fig. 2). While localized propleural structures are present in many lower and higher attine ant species, some species have filamentous bacteria covering the entire cuticle, occurring on non-localized foveae (typically in lower attines) or nonlocalized cuticular tubercles (higher attines). There are nevertheless interesting exceptions to the broad evolutionary patterns. First, the lower attine ant species *Cyphomyrmex laevigatus* does not have foveae; instead, the filamentous bacterium grows on porous tubercles that are morphologically similar to the tubercles found within the foveae of other species of "lower" attine ants (fig. S1E). Second, the "higher" attine species *T. zeteki* has foveae both on the propleural plate as well as distributed over the entire worker exoskeleton (fig. S1G). Third, two genera, *Sericomyrmex* and *Atta*, have no filamentous bacteria 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.

Focusing on the ant genus *Cyphomyrmex*, the shape, abundance, and size of foveae present on the propleura are highly variable across species. Some *Cyphomyrmex* spp. have a single large fovea on each propleural plate (e.g., *C. longiscapus* and *C. muelleri*), while other species have many smaller foveae covering the surface of the propleural plates (e.g., *C. costatus*, *C. rimosus*, and *C. cornutus*) (Fig. 2, fig. S1H), or totally lack foveae (*C. laevigatus*).

The presence of very diverse structures associated with the filamentous bacteria within the genus *Cyphomyrmex* suggests rapid evolution of the structures, possibly in response to coevolution with the filamentous bacteria. This may mirror the rapid evolution of modified cuticular structures of ambrosia beetles, where the modifications of specialized structures for housing mutualistic fungi (mycangia) are significant (S4, S5). As in attine ant structures, the widespread presence of mycangia in ambrosia beetles suggests an early origin in the Scolytinae; however, the location of mycangia in the otherwise extremely uniform beetles varies between beetle species and even between sexes within species (S4, S5). These rapidly modified structures in ambrosia beetles may have resulted during rapid species diversifications, but the functional significance is unknown. Likewise unknown is the causal reason for the diversification in cuticular structures for housing actinomycetes in attine ants, but this could be attributed to coevolution with the filamentous bacteria, possibly in response to a strong selective force imposed by the garden parasite *Escovopsis*.

While it is possible that the filamentous bacteria have multiple independent origins within the symbiosis, multiple lines of evidence support an early origin of the ant-bacteria mutualism. This evidence includes (i) the presence of structures to house and/or support the growth of bacteria across the attine ant tribe, (ii) the absence of these structures in non-fungus-growing ant genera, (iii) the specific and varied location of the bacteria on the cuticle, (iv) the diversity in cuticular structures, even within a single ant genus, and (v) the broad evolutionary patterns revealed by the examination of the specialized structures across the phylogenetic diversity of attine ants (see above, Fig. 2). A finding of multiple lineages of bacteria associated with the ants would perhaps not be surprising, as this would parallel the symbiosis between the ants and their cultivated fungi. Since the single origin of fungal cultivation by attine ants 50-65 million years ago, the ants have domesticated new strains of fungi for cultivation multiple times over their evolutionary history (S6).

Bioassays. Mean width (cm) \pm SE of zones of inhibition were 1.93 ± 0.30 in *Apterostigma*, 1.10 ± 0.30 in *Cyphomyrmex*, 1.67 ± 0.52 in *Trachymyrmex*, 1.63 ± 0.26 in *Acromyrmex*, and 2.17 ± 0.39 in *Atta*. The inhibitory abilities of *Escovopsis* by the

actinomycetes confirm previous findings that the actinomycetes produce diffusible metabolites that *Escovopsis* is susceptible to (S1, S2). Furthermore, the presence of zones of inhibition in all of the bioassay challenges suggests that the actinomycetes have evolved in parallel with typically infecting *Escovopsis* strains, in which the ability to suppress parasite growth by means of antibiotic production has been upheld.

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Supplementary Figure Legends

Fig. S1. Presence of morphological structures and glands for maintaining mutualistic bacteria in attine ants. **(A)** SEM of *C. longiscapus* showing a single fovea on a propleural plate. **(B)** SEM close-up of the fovea of *C. longiscapus* demonstrating the pores and microtubules. **(C)** TEM of the gland associated with the under surface of the cuticle, just beneath the fovea in *C. longiscapus*. **(D)** SEM of external openings of the fovea present on the exoskeleton of *C. longiscapus*. **(E)** SEM of the internal structures of two fovea present on the dorsal surface of *C. longiscapus*, revealing the presence of tubercles within the crypts. **(F)** TEM of the single-celled gland with duct cell just under the mesopleura in *Apterostigma* sp. **(G)** TEM of fovea and gland cells in the "higher" attine ant *T. zeteki*.

(H) SEM of opening in fovea on the propleuron in *C. cornutus*. Scale bars: as indicated or 10 μm .

Fig. S2. Growth of the filamentous bacterium directly on tubercles in fungus-growing ants. (A to D) SEM of 3-day-old (A and C) and 2-week-old (B and D) *C. costatus* workers revealing growth of the bacterium directly on tubercles within the foveae. (E to H) SEM of 3-day-old (E and G) and 7-day-old (F and H) workers of the leaf-cutter ant *A. octospinosus* revealing the growth of the filamentous bacterium directly on the tubercles. Scale bars: 10 μm .

Figure S1

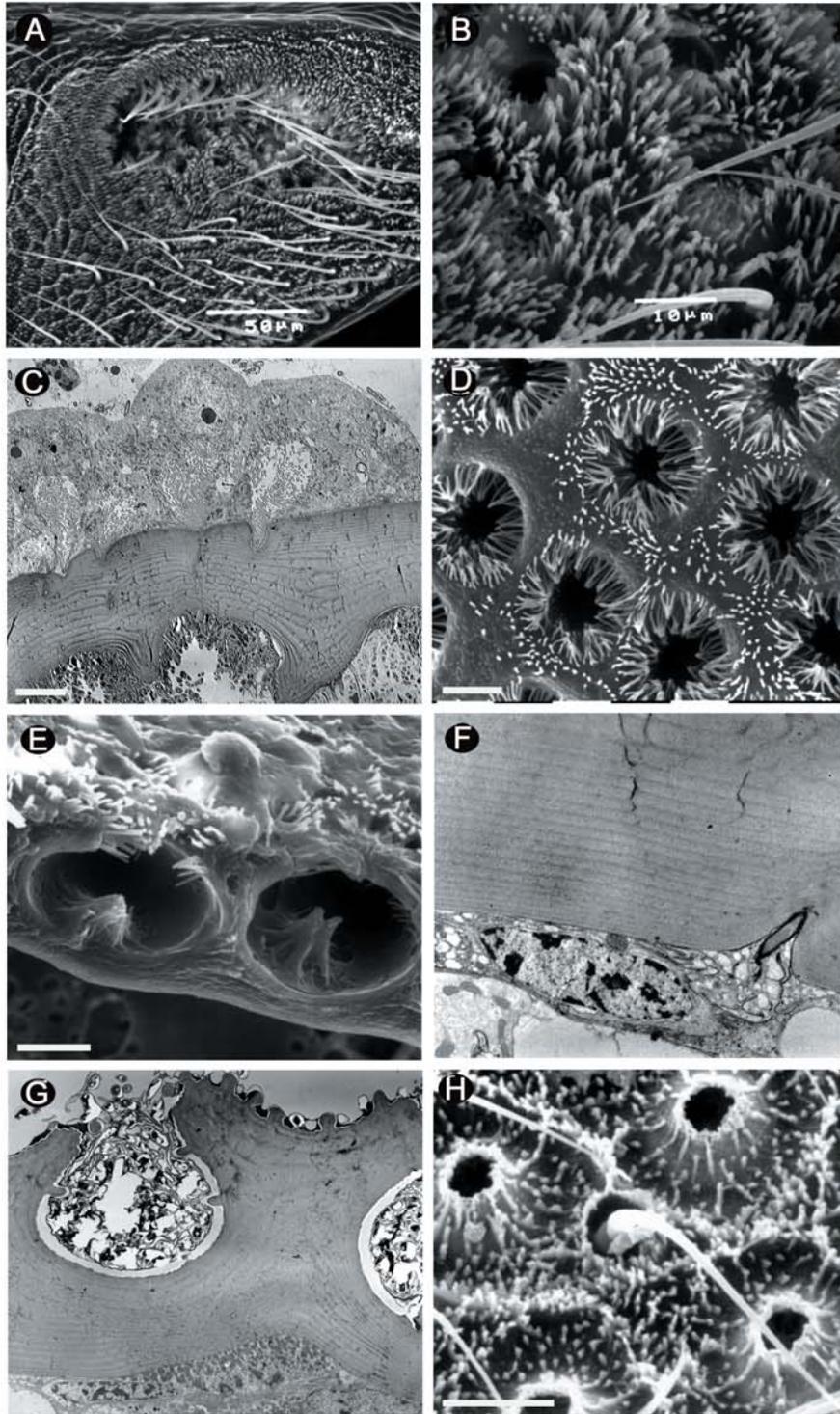


Figure S2

