Supporting Online Material for
Coevolved Crypts and Exocrine Glands Support Mutualistic Bacteria in Fungus-Growing Ants

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Published 6 January 2006, Science 311, 28 (2006)
DOI: 10.1126/science.1119744

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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:

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*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. echinatior, 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-µm 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 ~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) ± 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.

**References**


**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.