

information from our specimen reveals that they usually occur on the same radius as a type A branch and that they are similar to the radial bands of tissue that differentiate in the wood of living trees when latent primordia are produced¹⁵. These tissue bands are generally associated with the development of roots for vegetative propagation.

The type B branches of *Archaeopteris* represent a major innovation (Fig. 1c–d); five are recorded in the portion of trunk analysed. These laterals do not arise in the same ontogenetic spiral as the type A branches. They represent a new category of organ, of possibly adventitious origin, where the innermost part of its vascular trace, although close to the outer edge of the trunk primary vascular cylinder, apparently does not connect to it. Their sequence of production is irregular and currently unpredictable but the site where they originate is spatially determined, as they always occur on a radius next to the site of attachment of type A branches (Fig. 1c). Type B branches correspond to the largest traces running into the trunk, which suggests that they had a larger morphogenetic potential than the type A ones. Type B traces depart at approximately 30° from the horizontal and protrude on the outer surface of the trunk (Fig. 1d). They produce regularly arranged lateral appendages. The occurrence of short internodes in the basal part supports the view that type B organs had a delayed development. We interpret these branches as being long-lived structures that probably represent significant permanent constructional units of the tree architecture.

The earliest evidence of axillary branching was reported for Tournaisian seed plants of the family Calamopityaceae¹². Branches of *Calamopitys* are inserted in the axils of leaves but their traces connect to the closest vascular traces, not necessarily to the main stem, which suggests some period of branch primordium dormancy. The new *Archaeopteris* lateral branching syndrome, which involves the evolution of an extra type of branch and its spatial dependence on a leaf-type organ, apparently needs only a few positional adjustments to fit with the axillary branching of Calamopityaceae. It represents a major breakthrough from pseudomonopodial types of branching and may correspond to an evolutionary step towards the axillary branching of basal seed plants.

Several trunk fragments from Morocco bear the bases of large branches at wide angles. These show characteristic waves of undulating wood at their bottom points of attachment and collars of extra wood surrounding the sides and upper surface (Fig. 1b). The points of attachment of these perennial branches are reinforced by these external collars and by internal sockets for compressional and tensional load-bearing, just like modern tree limbs^{16–18} that grow heavier each year.

New information on branching patterns in *Archaeopteris* shows that this Devonian tree evolved most of the developmental features subsequently selected in the vegetative body of most derived seed plants. These features allowed the emergence of new growth forms which lived longer and could occupy space more efficiently, thereby increasing their fitness. They may partly explain the worldwide dominance of *Archaeopteris* forests on Late Devonian floodplains. □

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Fungus-growing ants use antibiotic-producing bacteria to control garden parasites

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The well-studied, ancient and highly evolved mutualism between fungus-growing ants and their fungi has become a model system in the study of symbiosis^{1–5}. Although it is thought at present to involve only two symbionts, associated with each other in near isolation from other organisms^{1–5}, the fungal gardens of attine ants are in fact host to a specialized and virulent parasitic fungus of the genus *Escovopsis* (Ascomycotina)⁶. Because the ants and their fungi are mutually dependent, the maintenance of stable fungal monocultures in the presence of weeds or parasites is critical to the survival of both organisms. Here we describe a new, third mutualist in this symbiosis, a filamentous bacterium (actinomycete) of the genus *Streptomyces* that produces antibiotics specifically targeted to suppress the growth of the specialized garden-parasite *Escovopsis*. This third mutualist is associated with all species of fungus-growing ants studied, is carried upon regions of the ants' cuticle that are genus specific, is transmitted vertically (from parent to offspring colonies), and has the capacity to promote the growth of the fungal mutualist, indicating that the association of *Streptomyces* with attine ants is both highly evolved and of ancient origin.

Because few organisms cultivate their own food, fungus-gardening by ants (Attini: Formicidae) is considered to be a major breakthrough in animal evolution⁷. These ants forage on a variety of substrates that they use for the cultivation of the vegetative mycelium of a fungus, their dominant food source. Fungus cultivation evolved apparently only once in the attines, over 50 million years ago, with the domestication of a fungus in the family Lepiotaceae (Agaricales: Basidiomycotina)^{3,4,8}. Other lepiotaceous lineages, and in one case a distantly related non-lepiotaceous basidiomycete, were

domesticated in subsequent evolutionary history⁴. The success of fungal cultivation by the attine ants is illustrated by the leaf-cutting genera, *Acromyrmex* and *Atta*, which are the dominant herbivores in the neotropics⁹.

Certain areas of the cuticle of fungus-growing ants are coated with what appears to the naked eye to be a powdery, whitish-grey crust (Fig. 1). This has been dismissed previously as a 'waxy bloom', implying that its aetiology was cuticular exudate¹⁰. Micromorphological and biochemical examination reveals that this coating is in fact formed from masses of a filamentous bacterium (actinomycete) of the genus *Streptomyces* (Fig. 2a; see Methods). Actinomycetes are mostly soil-dwelling organisms of great abundance and ecological importance that produce an array of secondary metabolites, many of which have specific antibacterial or antifungal properties^{11,12}. In fact, most antibiotics developed for human pharmaceutical use are actinomycete metabolites, many derived from the genus *Streptomyces*^{11,12}. In light of the unique biochemical properties of actinomycetes as a group, we proposed that the *Streptomyces* associated with fungus-growing ants may have an important function in this symbiosis, that of suppressing the growth of potentially devastating pathogens.

To ascertain the distribution and prevalence of this bacterium, both within and between species and genera of fungus-growing ants, we studied 22 species of attine ants representing 8 genera for the presence of the actinomycete. The bacterium was associated with all species studied, from the phylogenetically basal genera *Myrmicocrypta* and *Apterostigma* to the highly derived, leaf-cutting genera *Acromyrmex* and *Atta*. All 112 colonies from Panama sampled for the presence of the actinomycete in 1997 and 1998 showed this bacterial association. 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 *Streptomyces*, conceivably to facilitate the distribution of bacterial metabolites throughout the garden (Fig. 2, Table 1). In workers and queens of *Myrmicocrypta* and *Apterostigma*, the actinomycete mutualist occurs under the forelegs (Fig. 2b); in the more phylogenetically derived genera (Table 1), the actinomycete mutualist is most prominent on the laterocervical plates of the propleura (Fig. 2c), ventral, collar-like structures immediately posterior to the mouth-parts. The consistent association of the actinomycete with phylogenetically diverse attine ants, as well as its location on the ant integument (that is, conserved within species but unique



Figure 1 Photograph showing the presence of the third mutualist, *Streptomyces*, on the cuticle of *Acromyrmex octospinosus*.

between genera), indicates that this association is highly evolved and may be of ancient origin.

We studied the presence of the actinomycete on foundress queens (gynes) during their mating flights to determine whether, like the fungal mutualist, this bacterium is transmitted vertically between parent and offspring colonies. We examined 74 foundress queens and 15 males collected from throughout Gamboa, Panama, during the mating flight of *Acromyrmex octospinosus* on 9 May 1997. *Streptomyces* was present on the cuticle of all gynes examined, whereas it was absent from all males collected during the same mating flight. Examination of female and male reproductive individuals in natal gardens of *Trachymyrmex* cf. *zeteki* before mating flights revealed the presence of the cuticular actinomycete on females only ($n = 74$ ants, including 43 males, from 10 colonies). Because males do not participate in the founding of new colonies or

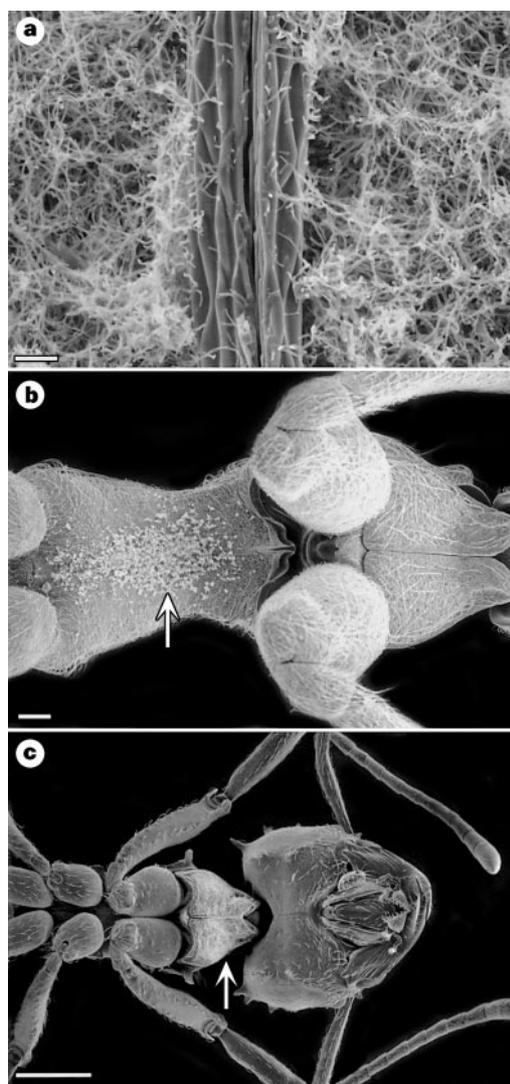


Figure 2 Scanning electron micrographs of fungus-growing ants, showing the location of *Streptomyces*. **a**, View of the filamentous growth form of the actinomycetous bacterium, showing the typical growth pattern and its thickness on the cuticle. Scale bar represents 10 μm . **b**, View of *Streptomyces* (arrow) under the forelegs of *Apterostigma* sp; this is the characteristic location of the bacterium in phylogenetically basal genera of the Attini. Scale bar represents 100 μm . **c**, Ventral view of a minimum worker of *Acromyrmex octospinosus*. The actinomycete-laden laterocervical plate (arrow) can be seen just below the head on the propleura of the ant. This is the characteristic location of the bacterium on the more phylogenetically derived genera of the Attini. Scale bar represents 500 μm .

Table 1 Location of *Streptomyces* on different genera of attine ants

Attine genera*	Under forelegs on propleura	Laterocervical plates of propleura
<i>Myrmicocrypta</i>	Yes	
<i>Apterostigma</i>	Yes	
<i>Mycocephurus</i>	Yes	Yes
<i>Cyphomyrmex</i>		Yes
<i>Trachymyrmex</i>		Yes
<i>Acromyrmex</i>		Yes
<i>Atta</i> †	Not visible on exoskeleton	Not visible on exoskeleton

* Attine genera listed from most phylogenetically basal to most phylogenetically derived^{8,23}.

† Although not visible on exoskeleton, *Streptomyces* was isolated repeatedly from intact *Atta* spp.

in tending the fungal garden, these data support the proposed role of the actinomycete in suppressing the growth of garden pathogens.

To determine whether the attine-associated actinomycete produces compounds with beneficial antimicrobial properties, we performed bioassays in which taxonomically diverse sets of fungi were challenged with attine *Streptomyces* isolates. *Streptomyces* isolates obtained from *Acromyrmex octospinosus* lacked detectable inhibitory effects on the growth of generalist saprotrophic fungi, entomopathogenic fungi, and other fungi commonly used for antibiotic screening. However, it showed potent inhibitory effects towards *Escovopsis*, a fungal genus (anamorphic Hypocreales: Ascomycotina)¹³ identified recently as a specialized virulent parasite of the attine fungal gardens⁶. The actinomycete completely suppressed spore germination of *Escovopsis* isolates in 25% of bioassays. Linear fungal growth in the remaining challenges was inhibited by $73.9 \pm 3.0\%$ (mean \pm s.e.m.), typically resulting in zones of inhibition larger than 30 mm (Fig. 3). Other bioassay challenges between the corresponding *Streptomyces* and *Escovopsis* from colonies of *Cyphomyrmex longiscapus*, *Atta colombica* and *Atta cephalotes* again resulted in significant inhibition of the growth of *Escovopsis*.

We tested growth-enhancing effects of the actinomycete on the basidiomycete mutualist in broth culture bioassays, using a *Streptomyces* isolate obtained from the phylogenetically basal attine genus *Apterostigma*. We observed significant increases in basidiomycete vegetative biomass in the presence of the actinomycete culture filtrate (vegetative biomass in presence of actinomycete culture filtrate averaged 47.9 ± 7.6 mg dry weight, versus 5.3 ± 2.4 mg dry weight for unamended controls; significant at $P = 0.0029$, Student's *t*-test). This increase in biomass may result from the production of growth-promoting compounds by the actinomycete (for example, vitamins, enzymes, and/or amino acids)^{14–16}.



Figure 3 Bioassay challenge between *Streptomyces* and *Escovopsis*, the specialized parasite of attine fungal gardens, associated with *Acromyrmex octospinosus*, illustrating the substantial zone of inhibition of fungal growth.

Several lines of evidence indicate that this newly discovered bacterial symbiont of the attine ants is a third mutualist in an ancient symbiosis among the ants, the domesticated fungi, the parasitic fungi, and the antibiotic-producing bacteria. First, this actinomycete is associated with all attine species and all colonies studied, representing the generic diversity of the Attini. Second, the actinomycete is transmitted vertically from parent to daughter nest, as is the fungal mutualist, and third, it promotes the growth of the fungal mutualist *in vitro*. Fourth, and most important, this *Streptomyces* produces highly potent antibiotics that selectively inhibit the growth of the garden parasite *Escovopsis*. Although many antibiotics developed for medicinal use have fairly broad-spectrum effects, it is likely that most fungicidal secondary metabolites produced by microbes evolved toward specific targets, such as competitors and pathogens^{17,18}. As the production of secondary metabolites is energetically costly and requires complex, genetically based biosynthetic pathways, its evolution and subsequent maintenance is presumed to impart a substantial selective advantage to the microbe¹⁷. Thus the production of antibiotics by the attine-associated *Streptomyces* that specifically target the growth of *Escovopsis* is compelling evidence that the bacterium is a highly evolved mutualist. The ants use this bacterium, because of its production of antibiotics, to treat microbial infection of their garden, and, in exchange, the ants disperse the actinomycete and appear to provide some form of nourishment for its growth.

Although the ant–fungus mutualism is often regarded as one of the most fascinating examples of a highly evolved symbiosis, it is now clear that its complexity has been greatly underestimated. The attine symbiosis appears to be a co-evolutionary ‘arms race’ between the garden parasite, *Escovopsis*, on the one hand, and the tripartite association amongst the actinomycete, the ant hosts, and the fungal mutualist on the other. The evolution of a mutualistic association between the attine ants and an actinomycete that suppresses parasites is perhaps not surprising. The benefits of such a symbiosis are illustrated by the paramount part played by therapeutic antibiotics in human biomedical history. Our results indicate that microbes and their metabolites may be key components in the regulation of other symbiotic associations between higher organisms, and thus a more detailed analysis of their functions promises to illuminate the general dynamics of symbiosis. Study of the presumably highly evolved chemical interactions between symbionts may provide valuable theoretical and practical insights regarding the identification, production and application of antibiotics^{19–21}. □

Methods

Identification of bacterium. To identify the bacterium, we used micro-morphological parameters as well as accepted biochemical criteria. We analysed the cell-wall fatty-acid methyl esters by gas–liquid chromatography²². The absence of tuberculosteric acid and related 10-methyl esters excluded the genera *Actinomadura* and *Nocardiopsis*, which are morphologically similar to *Streptomyces*.

Attines. The presence of actinomycete was determined in fungus-growing ant species from the canal region of Panama (112 colonies, 17 species) and the Napo province of Ecuador (25 colonies, 5 species). Attine genera studied included a representative sampling of both ‘lower’ (phylogenetically basal) and ‘higher’ (phylogenetically derived) attines, namely *Myrmicocrypta* (two species), *Apterostigma* (four species), *Mycocephurus* (two species), *Cyphomyrmex* (two species), *Sericomyrmex* (one species), *Trachymyrmex* (five species), *Acromyrmex* (three species) and *Atta* (three species).

Antibiotic-bioassay challenges. Bioassays were done in Petri dishes using an actinomycete isolate obtained from a phylogenetically derived attine species, *Acromyrmex octospinosus*. Saprotrophic fungi isolated from attine gardens, including taxa closely related to *Escovopsis*, were tested, as were generalist entomopathogens. We also assayed a diverse, representative set of fungi used for general anti-fungal screening. Finally, we challenged representative strains of the specialist garden parasite *Escovopsis*. Specifically, two strains of *Metarhizium anisopliae* and one strain of *Beauveria bassiana* were challenged

with actinomycete, as were the following common microfungi: *Absidia* sp., *Ascobolus crenulatus*, *Aspergillus fumigatus*, *Coprinus patouillardii*, *Cryptococcus albidus*, *Drechslera biseptata*, *Exophiala spinifera*, *Fusarium oxysporum*, *Mucor pyriformis*, *Penicillium* sp., *Pythium aphanidermatum*, *Schizophyllum commune*, *Sordaria fimicola* and *Trichoderma* sp.

Each *Streptomyces*–fungal challenge was replicated three times and done on Czapek yeast autolysate agar. The actinomycete was inoculated on Petri dishes and grown to a diameter of ~1.5 cm; fungal strains were then point-inoculated near the edge of the culture. Challenges were monitored every two days and growth inhibition of tested fungi was scored as a reduction of growth rate as compared with growth of fungal cultures in the absence of the *Streptomyces*, or as complete suppression of growth. We assayed possible antibiotic production specific to the specialized parasite *Escovopsis* in the same way that we assayed antibiotic production specific to other potential contaminants, except that each challenge to *Escovopsis* was replicated five times. Four strains of *Escovopsis* isolated from the gardens of different *Acromyrmex octospinosus* colonies in Panama in 1997 were tested against *Streptomyces*. We also studied the production of antibiotics specific towards *Escovopsis* in other attine species, including *Cyphomyrmex longiscapus*, *Atta colombica* and *Atta cephalotes*. The presence of a zone of inhibition in bioassays indicates first, the production of diffusible metabolites by the actinomycete, and second, the susceptibility of the test fungus to these compounds. As inhibition is dose dependent, the detection of partial inhibition implies the existence of a dose that could impart complete inhibition.

Growth-promotion bioassays. Broth cultures of the attine fungus isolated from an *Apterostigma* colony were grown with extracts from the *Streptomyces* isolated from this species. Actinomycete extracts were obtained by growing *Streptomyces* in Czapek yeast autolysate broth for 2 weeks and then passing the broth through a low protein-binding, sterilizing filter unit (Millipore, Millex) to remove bacterial biomass. We replicated each bioassay five times and used 50 ml Czapek yeast autolysate broth per bioassay.

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Relative reward preference in primate orbitofrontal cortex

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The orbital part of prefrontal cortex appears to be crucially involved in the motivational control of goal-directed behaviour^{1,2}. Patients with lesions of orbitofrontal cortex show impairments in making decisions about the expected outcome of actions³. Monkeys with orbitofrontal lesions respond abnormally to changes in reward expectations^{4,5} and show altered reward preferences⁶. As rewards constitute basic goals of behaviour⁷, we investigated here how neurons in the orbitofrontal cortex of monkeys process information about liquid and food rewards in a typical frontal task, spatial delayed responding⁸. The activity of orbitofrontal neurons increases in response to reward-predicting signals, during the expectation of rewards, and after the receipt of rewards. Neurons discriminate between different rewards, mainly irrespective of the spatial and visual features of reward-predicting stimuli and behavioural reactions. Most reward discriminations reflect the animals' relative preference among the available rewards, as expressed by their choice behaviour, rather than physical reward properties. Thus, neurons in the orbitofrontal cortex appear to process the motivational value of reward-outcomes of voluntary action.

Neurophysiological studies of behaving monkeys and rats show that neurons in the six-layered parts of orbitofrontal cortex process motivating events, discriminate between appetitive and aversive conditioned stimuli⁹ and are active during the expectation of outcomes¹⁰. Some orbitofrontal neurons may code the value of reward objects in losing their responses when animals become satiated on particular food items¹¹. Neurons in more caudal, three- and four-layered orbitofrontal regions process gustatory and olfactory information^{12–14}.

We investigated the motivational properties of orbitofrontal neurons in macaque monkeys during a spatial delayed-response task (Fig. 1). The position of a briefly presented instruction picture indicated the target of an arm movement, and its visual features predicted specifically which of two liquid or food rewards would be delivered for correct performance at the end of the trial. A subsequent uniform trigger stimulus provoked the movement to the remembered target. The reward was delivered after a brief delay during which the animal could expect the reward. Reaction times

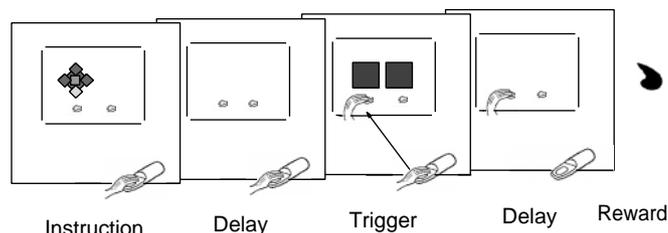


Figure 1 Spatial delayed-response task. An initial instruction picture indicates the left or right target of movement and the liquid or food reward that will be delivered at the end of the trial. Following a brief delay, two identical squares appear and the monkey moves its hand from the resting key to the left or right target lever indicated by the instruction. Correct performance is rewarded after a brief delay with a drop of liquid or a morsel of food.