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Symbiont recruitment versus ant-symbiont co-evolution in the attine ant–microbe symbiosis

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The symbiosis between fungus-farming ants (Attini, Formicidae), their cultivated fungi, garden-infecting *Escovopsis* pathogens, and *Pseudonocardia* bacteria on the ant integument has been popularized as an example of ant–*Escovopsis*–*Pseudonocardia* co-evolution. Recent research could not verify earlier conclusions regarding antibiotic-secreting, integumental *Pseudonocardia* that co-evolve to specifically suppress *Escovopsis* disease in an ancient co-evolutionary arms-race. Rather than long-term association with a single, co-evolving *Pseudonocardia* strain, attine ants accumulate complex, dynamic biofilms on their integument and in their gardens. Emerging views are that the integumental biofilms protect the ants primarily against ant diseases, whereas garden biofilms protect primarily against garden diseases; attine ants selectively recruit ('screen in') microbes into their biofilms; and the biofilms of ants and gardens serve diverse functions beyond disease-suppression.

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Introduction

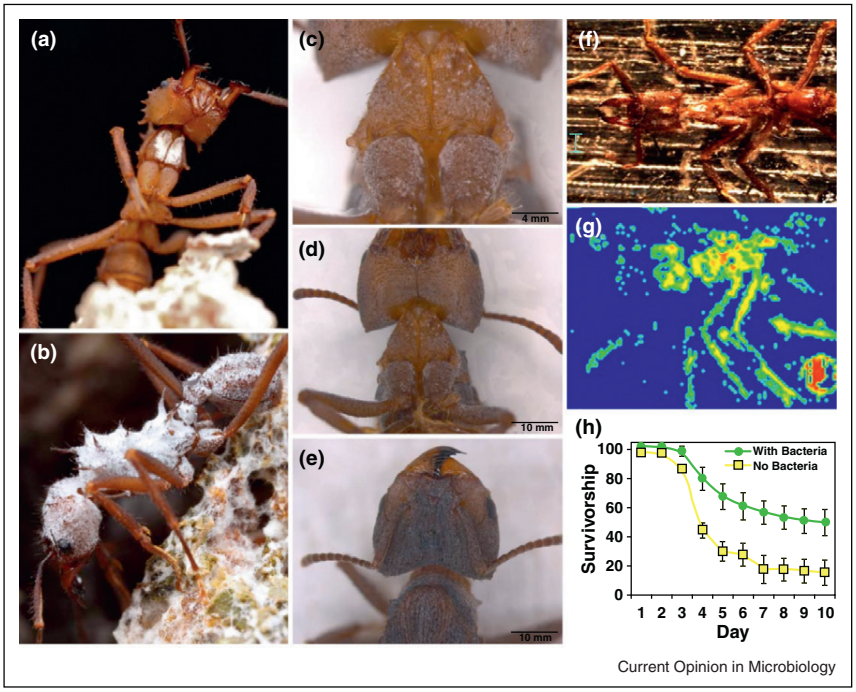
Among the diverse ant–microbe symbioses [1–6], the symbiosis between fungus-farming ants, their cultivated fungi, pathogens, and associated microbial biofilms has received disproportionate research attention [7–9,10•,11••]. This attention derived from appealing analogies between human agriculture and ant fungiculture, as well as from a fascination with the ants' fungicultural craftiness from which humans may perhaps learn tricks to manipulate microbial communities [7]. After nearly two decades of investigation into the diverse microbes associated with attine ants, attine microbial research has emerged rife with biases. Conclusions and assumptions that sustained the field for the past decade have failed replication in recent research. This review aims to find explanations for how it was possible that such captivating research could be so wrong for so long.

Attine fungiculture and the mis-specificity of *Pseudonocardia*

Fungus-farming ants (tribe Attini) grow fungi for food by manuring fungal gardens with various plant-derived substrate. The dominant biomass in an ant-garden is thought to be mycelium of the cultivated fungus, but the fungus is embedded in complex biofilms of competing, commensal, and mutualistic microbes (bacteria, yeasts, and non-cultivar filamentous fungi [7,12•,13,14••,15•,16,17••,18,19]). The garden microbiome is engineered partly by the behavior and secretions of the ants to optimize garden health and growth [7,12•,14••,19]. As in other animals, microbes also colonize the integument, the alimentary canal, and internal tissues of the farming ants (the ant microbiome) [2,3,20,21••,22]. Some attine ant species, but not all, accumulate visible whitish accretions on the integument from which actinomycete bacteria, yeasts, and other microbes can be isolated (Figure 1).

The first microbial report in 1999 on the integumental accretions of attine ants argued that the ants promote the growth of an antibiotic-secreting *Streptomyces* bacterium on their integument as a specific defense to suppress the microfungal garden parasite *Escovopsis* [13,23,24]. The initial identification of *Streptomyces* was soon thereafter revised to the actinomycete bacterium *Pseudonocardia* [25], although true *Streptomyces* were also known at that time to occur on attine ants (Electronic Supplemental Material). Because the presence of actinomycete bacteria and *Escovopsis* in attine nests was discovered at the same time [13,23], the co-discovery led to the immediate speculation that *Escovopsis* and the integumental microbes must be causally related. The earliest reports therefore argued that (a) attine ants promote the growth of mutualistic *Pseudonocardia* on their integument to derive bacterial secretions that specifically suppress *Escovopsis* diseases, while the integumental *Pseudonocardia* simultaneously stimulate growth of the cultivated fungus; (b) the integumental *Pseudonocardia* are antibiotically highly derived because they have been engaged in a co-evolutionary arms-race (Box 1) with *Escovopsis* since the origin of the attine ant-fungus mutualism 50 million years ago; and (c) *Escovopsis* failed to evolve effective resistance against *Pseudonocardia* because of some unknown disadvantage in the co-evolutionary arms-race [13,23,24,26]. This view on ancient, specific ant–*Pseudonocardia*–*Escovopsis* co-evolution still permeates the recent literature on attine ants

Figure 1



Diversity of microbial biofilms on the integument of three species of Acromyrmex fungus-farming ants. **(a)** Worker of *Acromyrmex echinatior*, showing a conspicuous white accretion on the propleural plate, ventrally on the anterior portion of the thorax. Such conspicuous white 'bibs' are found in some species of *Trachymyrmex* and *Acromyrmex* ants, but are absent in most other fungus-farming ant species. **(b)** Major worker of *Acro. octospinosus*, showing an extreme form of the integumental accretion covering the entire body. Such workers are relatively immobile and can be sluggish; a detrimental effect of the integumental accretion cannot be ruled out. **(c)** Filamentous growth on the propleural plate and the anterior coxae of an alate queen of *Acro. balzani*, a few days before leaving her natal nest for her mating flight. The filamentous growth extends all over the ventral **(d)** and dorsal **(e)** body surfaces, giving the appearance of a grayish dusting. **(f)** *Acro. echinatior* worker mounted for matrix-assisted laser desorption/ionization (MALDI) analysis, and **(g)** corresponding MALDI image revealing the distribution of the antibiotic valinomycin on the integument, with valinomycin concentration ranging logarithmically from blue (lowest) to red (highest) across the color spectrum. Valinomycin is secreted by *Streptomyces* bacteria occurring on the integument of *Acro. echinatior* [11••]. **(h)** Percent survivorship (±SEM) over ten days of *Acro. subterraneus subterraneus* workers pre-treated either with the antibacterial gentamicin ('no bacteria') or with sterile distilled water ('with bacteria'), then exposed to spores of the entomopathogenic fungus *Metarhizium anisopliae*. Gentamycin-treated workers without integumental bacterial biofilms have three times higher mortality when exposed to the pathogen than control workers with intact biofilms (figure redrawn from [21••]). Photo credit: Alex Wild (a and b); Tassio Brito de Oliveira (c–e); Souvik Kusari (f); Manoj Ghaste (g).

The original view of the function of the integumental biofilms [23,24,41,47] was developed largely from the study of *Acro. echinatior* and *octospinosus* (a and b); this view emphasized arms-race co-evolution of integumental *Pseudonocardia* bacteria that were thought to secrete co-evolved antibiotics ('herbicides') targeted by the ants specifically against *Escovopsis* garden-disease. Later findings invalidated key assumptions of this ant–*Pseudonocardia*–*Escovopsis* co-evolution model. The most recent studies reveal a protective role of the integumental biofilms against entomopathogenic diseases of the ants (f–h) [11••,21••]. Male ants, which do not help in maintaining the ant-gardens, can carry biofilms containing *Pseudonocardia*, *Streptomyces*, and other actinomycete bacteria [20,22], and workers of some attine ant species accumulate conspicuous biofilms, but the garden parasite *Escovopsis* does not infect gardens of these species [22]; such observations indicate that the integumental biofilms do not serve as specific antibiotic defense against *Escovopsis* in these species, but could serve other functions (Table 1). An emerging view is that, rather than protection against *Escovopsis* disease, the primary purpose of the integumental biofilms is protection of the ants against their own diseases.

[27–29], but has been critically reevaluated in the past two years [10•,11••,21••,30,31•,32••,33,34].

Popularization of ant–*Escovopsis*–*Pseudonocardia* co-evolution

>The portrayal of anciently co-evolved, pesticide-secreting, integumental *Pseudonocardia* bacteria was initially adopted with great enthusiasm by ant biologists and the general public, to the complete exclusion of other possible explanations of the integumental accretions (Table 1). For example, the National Science Teacher Association (NSTA) promulgated the ant–*Pseudonocardia*–*Escovopsis* association as an indisputable example of co-evolution [35]. In consultation with attine-ant researchers, NSTA developed a high-school teaching module to illustrate the coevolved nature of the *Pseudonocardia* defense (www.nsta.org/pdfs/virus/Virus-Activity3.pdf) and organized a corresponding museum exhibit that has been traveling for several years now through the USA. These educational aids present the attine ant–*Escovopsis*–*Pseudonocardia* symbiosis as an incontrovertible example of the concept of co-evolution,

Box 1 A primer of co-evolution

Co-evolution describes evolutionary change in populations of two interdependent species, where each population *changes adaptively and reciprocally* in response to changes in the population of the other species [62]. Prominent examples of co-evolution between species occur in strongly interacting antagonistic or mutualistic associations. *Antagonistic co-evolution* may occur between a fungus and a fungivore when a defensive adaptation in the fungus (e.g., toxin) arises in defense against the fungivore, and this defensive adaptation is then matched by the fungivore's counteradaptation (e.g., detoxification mechanism) to overcome the defense. Here, the toxin and the detoxification mechanism drive each other's evolution, which can continue as a prolonged arms-race co-evolution between defense (toxin) and resistance (detoxification). *Mutualistic co-evolution* may occur between pollinators and pollinated flowers, where a flower adaptation (e.g., flower spur length that selects for specific pollinators with long tongues) may evolve in response to adaptations of the pollinator to collect nectar efficiently (e.g., tongue length); here, the spur length and the tongue length drive each other's evolution.

Co-evolution requires sufficient *specialization* between interacting partners such that the partners exert selective influence on each other, and co-evolution can be accompanied by processes leading to *co-cladogenesis* (co-speciation and co-diversification in the partner lineages resulting in congruence in phylogenetic trees, specifically leading to clade-to-clade correspondences when comparing the phylogenetic relationships of one partner with those of the other partner). Specialization and clade-to-clade correspondences are not defining features of co-evolution, however, because other processes can also generate these patterns. For example, if a particular host recruits one kind of symbiont from free-living, non-symbiotic populations, whereas a second host recruits another kind of symbiont, the differential recruitment generates specialization and clade-to-clade correspondence in the absence of co-evolution. A frequent mistake is to interpret specialization (a requirement for co-evolution) and clade-to-clade correspondence (a possible consequence of co-evolution) as evidence for co-evolution, but these features represent insufficient evidence. The defining features of co-evolution are the evolutionary *modifications that arise reciprocally in interdependent lineages in response to modifications arising in the partnered lineages*. It is often difficult to document that (a) any such features exist, (b) these features are evolutionarily derived (modified from some ancestral state), and (c) these features arose as reciprocal responses to each other.

In many cases it is easy to show that a feature in one partner arose through interaction with one or several partner species (e.g., the attine integument appears to be evolutionarily modified to facilitate accumulation of microbial biofilms [63]), but it is difficult to find evidence that the partner species are evolutionarily modified (e.g., so far, no evolutionarily derived feature has been documented in any of the microbes residing in the attine integumental biofilms, where the derived feature clearly arose once the microbe associated with the ants; future biochemical and genomic analyses may reveal such features). Evolutionary modification is often unclear in facultative partners that live largely in a non-symbiotic, free-living state outside the symbiotic interaction and that are occasionally recruited by a host from free-living populations into the symbiosis (e.g., the diverse integumental microbes that are recruited by the ants from free-living microbial populations outside the ant–microbe symbiosis). Some of the most dominant host–symbiont mutualisms (e.g., nitrogen-fixing root-bacteria of plants, algal symbionts of polyps in the coral symbiosis) are based on such symbiont acquisition and continuous replacement of microbial partners that are recruited by hosts from free-living microbial populations; such recruitment occurs in the absence of any apparent co-evolutionary response in the acquired symbionts. The symbiont may not respond co-evolutionarily because mutualistic life is facultative for the symbiont, and symbiont evolution is therefore dictated largely by its free-living existence (i.e., symbionts form too fleeting associations with a host to adapt to mutualistic life). In such cases, a mutualism evolves to be obligate for the host in the absence of a co-evolutionary footprint on the symbiont [54••,64].

A second common mistake is failure to distinguish co-evolution from parallel evolution. For example, a set of interacting species in the same habitat may independently evolve to become more temperature resistant under climate change, but because the species evolve in parallel without direct influence on each other (i.e., modifications in one species do not drive modifications in other species), this is not a case of co-evolution. Instead, co-evolution requires a mechanistic link between interacting species that drives the reciprocal modifications [65,66]. Such mechanistic links can be difficult to analyze. For example, virulence evolution in the interaction between rabbits and the myxoma virus is frequently cited as an example of co-evolution, but it actually represents parallel evolution without co-evolution. Both rabbits and the virus evolve within an interaction (the virus infects rabbits), and rabbit populations can evolve resistance against the virus (i.e., rabbits become evolutionarily modified in response to virus infection), but the virus' impact on the rabbit host (the virus' ability to exploit host resources) evolves independent of any resistance arising in the host. Rather, the virus evolves a reduced fitness impact on the host (reduced exploitative ability) to prolong the life of the rabbit, which increases the virus' chance to be transmitted to a new host. It is important to note that the virus evolves reduced exploitative ability even in the absence of resistance evolution in the rabbit; that is, the virus can evolve reduced fitness impact on the rabbit host *independent* of any evolutionary change in resistance of the host. Consequently, resistance of the rabbit and the exploitative ability of the virus are not mechanistically linked and rather evolve in parallel in the same interaction, but virus and rabbit do not co-evolve (the evolutionary modifications arising in rabbit and virus do not drive each other, thus not fulfilling the definition of co-evolution).

complementing canonical examples of other key concepts in evolution, such as natural selection in Darwin's finches, or descent with modification in the fossil record [35].

Co-evolution that isn't

A series of recent studies eroded all of the original evidence for ant–*Escovopsis*–*Pseudonocardia* co-evolution. Specifically, the antibiotic-specificity reported originally for a single bacterial isolate from an *Acromyrmex* ant ('lacked detectable inhibitory effects on the growth' of 17 test fungi in a culture-confrontation plate assay [23]) could not be verified in any subsequent study [20,32••,33,34,36••,37,38•]. Moreover, the growth-enhancing effect on the ant-cultivated fungus reported originally for a single bacterial isolate from an *Apterostigma* ant ('significant increases in ... [ant–cultivar] biomass in the presence of the actinomycete culture filtrate' [23]) has yet to be replicated [20,40]. Lastly, the original, antibiotically-potent *Pseudonocardia* isolate from *Acromyrmex* had apparently been tested only against 17 non-cultivar test fungi but not against the ant-cultivated fungus, which should have shown the severe growth-inhibition of ant-cultivars that could be readily documented in subsequent experiments [20,40]. There exists at present, therefore, no evidence that the ant-associated integumental microbes are evolutionarily modified, nor any evidence that any specific properties

Table 1

Possible functions of integumental microbial biofilms in fungus-gardening ants

Antibiotic protection	
Against ant diseases	Protection against entomopathogenic fungi [11,20,21••,38•] (Figure 1)
Against garden diseases	Protection against *Escovopsis* disease [13,23], *Syncephalastrum* disease [15,20], endophytic garden invadors [67], or many other potential diseases of gardens [16]
Against parasites	Protection against ectoparasites (e.g., mites) or endoparasites (e.g., phorid flies)
Chemical deterrence of predators	Protection of reproductive females during the risky mating flight or during foraging at the solitary nest-founding stage
Colonization resistance of the ant integument	Reduced colonization of the ant integument by detrimental microbes through nutrient depletion, through biofilm-induced stimulation of innate immune responses, or through direct inhibition (e.g., resistance against the many biofilm microbes with which the ants are constantly shoulder-rubbing in their garden, or even against the cultivated fungus, which can grow on the integument of both larval and adult attine ants [68])
Detoxification	Degradation of natural toxins secreted by the cultivated fungus or the garden biofilms
Enzyme secretion	Conversion of garden metabolites accumulating on the integument during gardening activities
Immune priming	Stimulation of facultative immune responses
Stress tolerance	Desiccation resistance; tolerance of carbon-monoxide or carbon-dioxide accumulating in the deep underground nests (most of these waste gases are produced by the fungus gardens); amelioration of redox stresses
Nutrients	Growth of microbial biofilms as food supplement [17••,69]
No function or pathogenicity	Detrimental or neutral effects of the biofilms on ant fitness cannot be ruled out *a priori*, and these alternative hypotheses serve as null hypotheses unless a beneficial function can be demonstrated; biofilms may be beneficial only under specific conditions, but otherwise are neutral or detrimental

The functions are not mutually exclusive, and the same functions could also apply to garden biofilms, nest-wall biofilms, or microbial communities in garden-dumps [18]. In over a decade of work on the attine integumental biofilms, no comprehensive list of possible functions of the integumental biofilms has been generated, and only two of these functions (antibiotic protection against ant diseases or against garden diseases) have been considered so far.

of *Pseudonocardia* evolved in response to co-evolutionary modification of the ants or *Escovopsis*. This erodes the presumed evidence for co-evolution (Box 1), although biochemical or genomic studies may uncover such evidence in the future [9,30].

A breakthrough in elucidating the attine ant–microbe symbiosis was the discovery by Kost *et al.* [36••] that workers from a single nest of the attine ant *Acromymrex octospinosus* (the same ant species also studied originally by [23,24,41,42]) carry a diversity of actinomycete bacteria on the integument, and that the antibiotic activities of actinomycetes that can be isolated from the ant integument are not superior at suppressing *Escovopsis* than actinomycetes that can be isolated from any random non-fungus-growing ant [36••]. *Streptomyces* and *Pseudonocardia* have now been repeatedly found to coexist in the same integumental biofilms on *Acromyrmex* [11••,32••,43•] and in the biofilms of other attine ants [22,30], but a great diversity of actinomycetes can actually be isolated from the integument of various attine ant species, including for example *Amycolatopsis* [20,22], *Tsukamurella* [30,32••], *Nocardiopsis* [32••], *Propionicimonas* [43•], and *Kribbella* ([22]; *Kribbella* is identified as *Nocardioides* in [27]). These observations suggest that the biofilms are taxonomically more complex than the integumental monoculture of a single *Pseudonocardia* strain postulated by some [27,33,42]) and that microbes such as *Streptomyces* play more important roles [11••,32••,38•,43•,44] than originally thought when known *Streptomyces* isolates from attine ants were disregarded as unimportant contaminants (Electronic Supplemental Material).

A second rectifying insight was that *Pseudonocardia* from the integument of attine ants have generalized, broad-spectrum antibiotic activities [20,32••,36••,39], comparable to the activities of environmental (free-living) actinomycete bacteria; this added to the evidence that the integumental *Pseudonocardia* are not antibiotically specialized to target only *Escovopsis*, and that they are therefore not evolutionarily derived as postulated by the original co-evolution model [23]. Third, in two studies [32••,43•] in which both *Pseudonocardia* and *Streptomyces* were isolated from the integument of workers from the same ant colony, *Streptomyces* isolates inhibited *Escovopsis* more strongly than *Pseudonocardia* isolates. In fact, *Pseudonocardia* isolated from the attine integument can be completely ineffective or only minimally effective against *Escovopsis* [20,32••,33,38•,43•,45]. These latter observations do not necessarily refute co-evolution, because some *Escovopsis* may overcome the *Pseudonocardia* defense in the postulated evolutionary arms-race [45], but it is actually not possible to make useful antibiotic predictions to test the hypothesis of arms-race co-evolution, because both effectiveness and ineffectiveness can be consistent with arms-race co-evolution. Specifically, universal effectiveness against *all Escovopsis* was used originally as evidence supporting co-evolution by [23,24], but ineffectiveness of some *Pseudonocardia* strains

against *Escovopsis* was likewise used as evidence for co-evolution by [45], so co-evolution actually cannot be tested with this approach.

Fourth, phylogenetic analyses have revealed that all ant-associated *Pseudonocardia* strains are closely related to, or are identical to, free-living *Pseudonocardia* species [30,31•,33], indicating frequent acquisition of these microbes from environmental sources [30,31•]. These phylogenetic patterns are inconsistent with the long-term vertical inheritance between ant generations that was predicted by the ant–*Pseudonocardia*–*Escovopsis* co-evolution model to lead to derived clades of ant-associated *Pseudonocardia*. Fifth, as already mentioned, the antibiotics secreted *in vitro* by ant-associated *Pseudonocardia* kill or severely inhibit the ant-cultivated fungi from corresponding ant nests [20,40]; this contradicts the original finding of a growth-enhancing effect on the ant-cultivated fungus [23], although this inhibitory effect can be absent or attenuated *in vivo* in natural nests [40]. If some of the ants indeed derive *Escovopsis*-suppressing antibiotics from their integumental microbes, they would need to target these secretions judiciously to *Escovopsis*-infected portions of the garden, rather than apply antibiotics preventively across the entire garden, otherwise the ants harm the growth of their gardens [20,46•].

Emerging views: ant microbiomes defend against ant diseases, garden microbiomes defend against garden diseases

Because all of the early findings supporting ant–*Pseudonocardia*–*Escovopsis* co-evolution cannot be replicated, and furthermore, because the initial claim of clade-to-clade *Escovopsis*–cultivar specificity [47] likewise cannot be verified [48,49; Electronic Supplementary Material], the most recent literature questions the general importance of the integumental biofilms as specific defense against *Escovopsis* garden-disease [11••,20,21••,22,30,32••,36••,38•]. Instead, recent investigations have begun to explore alternate functions of the integumental biofilms, such as protection against ant diseases ([11••,20,21••,38•]; Figure 1), which may be a more primary function than any indirect defense against garden disease.

A second emerging realization is that diverse microbes in gardens appear to contribute to defense against *Escovopsis* and other garden diseases [14••,15•,34,38•]. *Escovopsis*-suppressing *Burkholderia* bacteria [14••], yeasts [15•], and *Streptomyces* [34,38•] have been isolated from the biofilms in leafcutter gardens, and a *Streptomyces* isolate from a garden biofilm of *Acro. octospinosus* most strongly inhibited *Escovopsis* through candicidin secretion, but this *Streptomyces* secretion did not harm the ant-cultivated fungus *in vitro* [34,38•].

Except for the study by Schoenian *et al.* [11••] (Figure 1f and g), all of the above antibiotic tests were conducted *in vitro*, and it is possible that antibiotic effects observed in *in vitro* experiments could differ from *in vivo* conditions of natural ant nests. However, it is also possible that the recently documented antibiotic interactions are representative for natural attine nests, that therefore the integumental biofilms primarily protect the ants against their own diseases ([11••,21••]; Figure 1), and that the integumental biofilms are largely irrelevant for *Escovopsis* defense (or perhaps do so in only some ant species). Instead, a combination of the ants' gardening behaviors, the antibiotic secretions of the ants and the cultivated fungi, as well as the auxiliary microbes in the garden biofilms (some of them secreting several antibiotics as a kind of 'combination therapy' [34,50]) are primarily responsible for disease suppression in gardens, as first suggested by [14••] and further elaborated by [7].

Evolutionary stability under symbiont recruitment versus host–symbiont co-evolution: the relative importance of partner-fidelity feedback, partner choice, and multi-level selection

The recent revision of the co-evolutionary interpretation of ant–*Pseudonocardia*–*Escovopsis* interaction triggered a corresponding revision of the evolutionary mechanisms thought to prevent the invasion of undesirable microbial partners into the symbiosis (e.g., invasion of self-serving microbial mutants arising within the symbiosis; or *de novo* acquisition of undesirable symbionts). The original co-evolutionary model emphasized partner-fidelity feedback [51] under long-term vertical inheritance of the integumental microbes across ant generations [23,24,26]. Recent models encompass frequent acquisition of novel microbial symbionts, emphasizing either higher-level selection on ant-*Pseudonocardia* combinations [46•] or selective recruitment into the biofilms through mechanisms of partner choice [10•,30,36••,51,52••]. Like many other ecologically dominant host–microbe mutualisms operating in the absence of co-evolution, the attine microbiome symbioses are open, and the ants might forge associations with beneficial microbes through complex processes of selective recruitment, selective rewarding, sanctioning, and/or purging of the microbial symbionts [51,52••,53•,54••]. Beneficial microbes that are recruited into the integumental biofilms [10•] or into the garden biofilms [7,14••,38•] could be acquired by such mechanisms of partner choice [51,52••,53•,54••], and future investigations into selective recruitment and selective retention of beneficial microbes promise the most fruitful insights into the nature of attine biofilms.

Higher-level selection most likely operates on ant–cultivar combinations [7] and by extension also perhaps on ant–cultivar–bacteria combinations [46•], but because the bacterial components are more frequently acquired and substituted than the cultivated fungi, such higher-level selection would require significant differential colony

survival or colony reproduction that is correlated with phenotypic variation (e.g., colony resistance against *Escovopsis*) of ant–cultivar–bacteria combinations. Such natural selection on ant–cultivar–bacteria combinations could operate at the nest-founding stage (more than 99% of all newly founded nests perish), which is a stage when *Escovopsis* is thought not to infect gardens [13]. It is thus unclear whether sufficient higher-level selection is possible to drive any kind of evolutionary response in ant–cultivar–bacteria combinations to defend against *Escovopsis*. Lower-level processes, such as selection on ants, on the cultivated fungi, or on the garden-associated microbiomes that are propagated differentially by the ants [7,14••] therefore would seem to be more important in shaping defenses against garden pathogens than higher-level selection.

Escovopsis-suppression *in vivo* by *Pseudonocardia*: need for more replicated experimentation

At present, observations from three studies support a possible role of the integumental microbes in *Escovopsis*-suppression. First, when scraping off integumental accretions from the propleural plate of *Acro. octospinosus* (a procedure that may also harm the ants), gardens tended by these scraped ants become more susceptible to *Escovopsis* infection [41]. Second, protection against *Escovopsis in vivo* can be predicted from *Pseudonocardia–Escovopsis* interactions observable *in vitro* [45]. Specifically, *Pseudonocardia* strains that strongly inhibit *Escovopsis in vitro* reduce garden loss due to *Escovopsis* by about 34% in experimental subcolonies, each consisting of a garden fragment that is tended by two major workers of *Acro. octospinosus* with maximum integumental biofilms (see Figure 1b); however, *Pseudonocardia* strains that inhibit *Ecovopsis* moderately *in vitro* are as ineffective *in vivo* as *Pseudonocardia* strains that are completely ineffective *in vitro* [45]. Third, for a sample of 14 *Pseudonocardia* strains from attine ants and 7 non-symbiotic *Pseudonocardia* strains obtained from culture collections, attine *Pseudonocardia* are better (by about 20%) at inhibiting *Escovopsis* than the non-symbiotic *Pseudonocardia* [33]. Although this comparative study does not correct for confounding effects of phylogeny (non-symbiotic *Pseudonocardia* most closely related to the attine *Pseudoncoardia* are as effective at suppressing *Escovopsis* than the attine *Pseudonocardia*; Figure ESM4 in [33]), and although antibiotic properties may have been lost in some of the tested non-symbiotic strains during decades of lab cultivation, this result is consistent with the hypothesis that ant-associated *Pseudonocardia* may be selectively recruited for the purpose of *Escovopsis* defense, or that *Pseudonocardia* evolve and maintain effective *Escovopsis* defense inside the symbiosis. Because other key findings reported originally for ant–*Pseudonocardia–Escovopsis* interaction could not be verified, replication of the above three studies on *Acromyrmex* ants and replication with other attine ant genera will help reestablish confidence in the postulated ant–*Pseudonocardia–Escovopsis* interactions. Such replication of key experiments should adopt the prudent guidelines for verification that have been successful outside of microbial ecology ('can other scientists access the data and protocols, repeat the analyses, and get the same results?' [55,56]).

Whereto next in attine microbial ecology?

After more than a decade of high visibility, attine microbial ecology is currently undergoing a sobering correction [10•,11••,21••,32••,34], as early findings supporting ant–*Pseudonocardia–Escovopsis* co-evolution have failed verification in recent studies. Some of the missteps in need of correction derived from an improper understanding of co-evolution (Box 1), some missteps from overextended inference from small sample sizes (Electronic Supplemental Material), and some missteps from incomplete hypothesis testing (Table 1). Future biochemical and genomic studies [9,30] may be able to provide the missing evidence for ant–*Pseudonocardia–Escovopsis* co-evolution. It is also possible there exist specific ant populations, such as *Acro. octospinosus* studied in Panama, for which *Escovopsis*-suppression by integumental *Pseudonocardia* may be crucial, whereas such suppression is unimportant in other ant populations of the same or other ant species. However, complete absence of any kind of co-evolutionary interactions between attine ants, their integumental microbes, and their *Escovopsis* pathogens is plausible, as many ecologically dominant host–microbe symbioses depend on recruitment of specific microbes [51,52••] and on continuous symbiont turnover, thus functioning well in the absence of co-evolutionary interplay [53•,54••].

To resolve the relative importance of selective microbe-recruitment versus co-evolution in attine microbial ecology, what is needed is (a) replication [55,56] of key experiments testing for ant–*Pseudonocardia–Escovopsis* co-evolution in and beyond *Acro. octospinosus*; (b) characterization of the diverse roles of the microbes in the integumental biofilms (does microbial competition in the biofilms generate selection for antibiotic innovation that is beneficial or detrimental to the ants?); (c) elucidation of the complexity of host–microbe interactions (is *Pseudonocardia* detrimental to the ants under some conditions; could the presumed parasitic black yeasts [57] on the attine integument actually serve beneficial functions?); (d) analysis of the gene-exchange network [58,59] among the ant-associated microbes (are genetic transfers facilitated by viruses or plasmids?) and whether such gene exchange influences biofilm properties; (e) characterization of the possible roles of *Pseudonocardia* in other symbioses, including the ecological links between those *Pseudonocardia* types that are endosymbionts of leaves and roots and that also associate with attine ants [31•]; (f) whole-genome sequencing of attine-associated

microbes [32••,60] and their closest free-living relatives to identify functional traits that may have arisen in association with the ants; and (g) in addition to antibiotic protection, testing of alternative functions of the integumental biofilms (Table 1) including non-adaptive functions.

Conclusions and perspectives

Scientific missteps can impose significant cleanup costs on a research community until errors are purged [61]. In the case of attine research that recently failed to replicate key assumptions of ant–*Pseudonocardia*–*Escovopsis* co-evolution, the cleanup costs faced now by the research community are difficult to estimate, particularly because the claim of attine–*Pseudonocardia*–*Escovopsis* co-evolution was allowed to be popularized [24,28,35] without replication of key findings and without testing of alternate hypotheses. For the historians of science, therefore, it may be interesting to ask what views would have been popularized if the attine integumental bacteria had been discovered not coincident with the discovery of *Escovopsis* garden-disease, but coincident with the discovery of some other problem organism in attine nests, such as mites or entomopathogens. Would a view have been popularized that the integumental bacteria are a specialized, co-evolved defense against one of these other problem organisms? If so, the past decade of attine ant–microbe research may represent less a textbook example for elucidating host–microbe mutualism, but a textbook example for how readily scientists and the public can be misled by inference from small samples sizes (Electronic Supplemental Material), and how captivating popularizations can prejudice scientific inquiry.

Conflict of interest

No conflicting interests.

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Papers of particular interest, published within the period of review, have been highlighted as:

- • of special interest
- •• of outstanding interest

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.mib.2012.03.001.

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Electronic Supplementary Material

Symbiont recruitment versus ant-symbiont co-evolution in the attine ant-microbe symbiosis

Ulrich G. Mueller

Biases

Several biases led to premature conclusions that misguided investigations into the attine ant-microbe symbiosis. These biases explain why such captivating research could be so wrong for so long.

Small sample sizes and unrepresentative samples biased conclusions: Key studies in attine-microbe research based conclusions on small sample sizes:

(1) The first study [Hinkle et al. 1994] on ant-cultivar co-evolution based conclusions on phylogenetic information from 5 ant-cultivated fungi. Phylogenetic relationships of these five fungi were topologically congruence with those of the corresponding ant species, and this clade-to-clade correspondence supported the conclusion of strict co-cladogenesis and tight ant-fungus co-evolution. Studies relying on larger sample sizes [Chapela et al. 1994; Mueller et al. 1998; Mikheyev et al. 2010] showed much more complex ant-fungus associations (e.g., ants frequently exchange fungi between each other; novel fungal types are regularly imported into the symbiosis by some ants), eroding the evidence that originally suggested tight ant-cultivar co-evolution.

(2) Likewise, the first study [Currie et al. 2003] testing for clade-to-clade correspondences between *Escovopsis*, attine ants, and their cultivated fungi relied on very few taxa per *Escovopsis* clade. In some cases, *Escovopsis* taxa were included that were known to be unrepresentative for the ant gardens from which they were isolated. For example, two of the so-called white-spored *Escovopsis* (a basal clade of *Escovopsis* [Gerardo et al. 2006]) were included in [Currie et al. 2003] as representatives for strains associated with *Apterostigma* ants (a basal clade of attine ants). White-spored *Escovopsis* actually rarely infect *Apterostigma* gardens (prevalence of 7.7% [Gerardo et al. 2006]), whereas the much more prevalent brown-spored *Escovopsis* (prevalence of 63.5% in *Apterostigma* gardens [Gerardo et al. 2006]) were not included in the first phylogenetic analysis [Currie et al. 2003]. Because brown-spored *Escovopsis* from *Apterostigma* are the closest relatives of brown-spored *Escovopsis* from leafcutter ants (a very derived clade of attine ants), the omission of brown-spored *Escovopsis* from *Apterostigma* therefore led to the incorrect conclusion of perfect clade-to-clade correspondence that supported the original conclusion of co-cladogenesis and co-evolution [Currie et al. 2003]. More comprehensive sampling (i.e., inclusion of the representative brown-spored *Escovopsis* from *Apterostigma*) would have shown some of the true topological incongruences that exist between the phylogenies of attine ants, their cultivars, and associated *Escovopsis* diversity (e.g., Figure 3 in [Gerardo et al. 2006]), invalidating the argument of strict clade-to-clade co-evolution. More recently, a comprehensive population-genetic survey failed to find genotype-to-genotype correspondences predicted under cultivar-*Escovopsis* co-evolution (Gerardo & Caldera 2007), and ongoing work [Katrin Kellner in preparation; Andre Rodrigues personal communication; Ulrich Mueller in preparation] uncovered a number of cultivar-*Escovopsis* associations in field nests that further invalidate the tidy clade-to-clade correspondences reported in the initial phylogenetic analysis by [Currie et al. 2003]. As above, initial reliance on a small sample of sometimes unrepresentative isolates led to the premature conclusion of clade-to-clade correspondences and tight co-evolution.

(3) Another choice of an unrepresentative sample occurred in the first study that investigated the antibiotic properties of the attine integumental microbes [Currie et al. 1999]. Although more than 22 actinomycete strains had been isolated from 22 attine ant species, the key conclusion of [Currie et al. 1999] regarding antibiotic specificity and co-evolution was based on tests with only one single *Pseudonocardia* isolate (Cameron R. Currie, personal communication). That *Pseudonocardia* strain (from *Acro. octospinosus*; Figure 3 in [Currie et al. 1999]) inhibited all *Escovopsis* isolates tested, but showed “no detectable inhibitory effects on the growth” of 17 test fungi [Currie et al. 1999]). No subsequent study has found a *Pseudonocardia* isolate with such specific antibiotic activity inhibiting only *Escovopsis* (but inhibiting no other fungi), and all of the dozens of attine-associated *Pseudonocardia* isolates that have been tested subsequently showed at least some non-specific

antibiotic properties [Kost et al. 2007; Haeder et al. 2009; Sen et al. 2009; Oh et al. 2009; Barke et al. 2010; Cafaro et al. 2011; Seipke et al. 2011]. Likewise, a second, actinomycete bacterium isolated from the integument of an *Apterostigma* ant showed a growth-enhancing effect on the ant-cultivated fungus from *Apterostigma* ("significant increase in … [ant-cultivar] biomass in the presence of culture filtrate" [Currie et al. 1999]), but all subsequent tests showed strong growth inhibition or lethal effects of integumental actinomycetes on the corresponding ant-cultivated fungi [Sen et al. 2009; Poulsen & Currie 2010]. (The identity of the growth-enhancing actinomycete tested in Currie et al. [1999] is unclear, i.e., it may not have been a *Pseudonocardia* bacterium, but *Streptomyces* [Mueller, Currie, Schultz 2001]). Moreover, the single, antibiotically-potent *Pseudonocardia* isolate from *Acromyrmex* [Currie et al. 1999] had apparently been tested only against 17 non-cultivar test fungi but not against the fungus cultivated by *Acromyrmex*; such an experiment should have revealed already a decade ago the severe growth-inhibition of ant-cultivars that could be readily documented in subsequent studies [Sen et al. 2009; Poulsen & Currie 2010]. In sum, testing of a larger sample of representative *Pseudonocardia* isolated from the integument should have shown that the integumental *Pseudonocardia* are antibiotically non-specific (i.e., the integumental *Pseudonocardia* inhibit not only *Escovopsis*), and that they rarely (if at all) enhance the growth of the corresponding ant-cultivated fungi. These insights would have invalidated already a decade ago any conclusion regarding ant-actinomycete-*Escovopsis* co-evolution, and would have ruled out the ant-*Pseudonocardia*-*Escovopsis* symbiosis as an incontrovertible example of co-evolution useful for teaching of key concepts in evolution [Diamond 2006].

Isolation biases underestimated biofilm diversity and biofilm properties: The most comprehensive study to date [Ishak et al. 2011] found that, as expected, the low-carbon chitin medium used traditionally to isolate *Pseudonocardia* from attine ants [Cafaro & Currie 2005; Sen et al. 2009; Cafaro et al. 2011] overestimates abundances of autotrophic bacteria. Autotrophic metabolism is known for *Pseudonocardia dioxanivorans* (which can fix carbon-dioxide [Sales et al. 2011]), and *P. carboxydivorans* (which can fix carbon-monoxide [Park et al. 2008]). *P. carboxydivorans* is the closest relative of the *Pseudonocardia* types frequently isolated from *Acromyrmex* and *Trachymyrmex* ants [Mueller et al. 2008, 2010; Cafaro et al. 2011; Ishak et al. 2011]), but the metabolism of ant-associated, symbiotic *Pseudonocardia*, which is currently completely unknown, may not be predictable from phylogenetic proximity to non-symbiotic *Pseudonocardia*. Better evidence exists that the low-carbon medium used frequently to screen the attine-ant microbiome underestimates microbial diversity [Zucchi et al. 2010; Ishak et al. 2011]. For example, *Solirubrobacter* dominates the microbiomes of the attine ant *Trachymyrmex septentrionalis* in culture-independent 16S-amplicon 454-sequencing screens, but *Solirubrobacter* could not be isolated in culture-dependent screens (over 900 isolates) of the same ant samples [Ishak et al. 2011]. These observations imply that some dominant components of the integumental biofilms of attine ants may not be readily culturable with the standard isolation methods used so far, while the abundances of autotrophic bacteria are likely overestimated.

It is presently unknown whether a diversity of microbes exists inside the integumental modifications that are thought to sequester and nourish the integumental microbes [Currie et al. 2006], or whether the integumental biofilms are layered, with some bacterial types dominating the microbial communities in the integument-adhering biofilm layers, and with a greater diversity of microbes coexisting in the more superficial layers that are most likely to recruit microbes dynamically from the environment. Future studies will need to elucidate the properties and possible functions of any such layering in the integumental biofilms.

Hypothesis-testing bias: Research into the integumental microbiomes of attine ants focused entirely on protection against the garden parasite *Escovopsis*. This focus was motivated by the belief that there exists only one microbe on the ant integument (one *Pseudonocardia* strain), and that this microbe is engaged in a co-evolutionary arms race with *Escovopsis*. Other possible roles (Table 1, main article) were not considered because the antibiotic specificity reported originally [Currie et al. 1999] could only be the outcome of a specific co-evolutionary interaction, but not the outcome of interactions with diverse problem-microbes in attine nests. Only in the past few years were alternative hypotheses explored for the first time [Kost et al. 2007; Sen et al. 2009; Barke et al. 2010, 2011;

Schoenian et al. 2011; Mattoso et al. 2012]. Additional potential functions of attine biofilms (Table 1, main article) have yet to be explored.

Microbial mutualists can serve potentially many functions (Table 1), many of them not mutually exclusive (e.g., protection against entomopathogens, protection against mite parasites, and protection against predators). Of the multiple function, the function for which it is easiest to obtain partially supportive evidence is antibiosis (*in vitro*, most microbes show at least some weak inhibitory activity – often accidentally - against test fungi), and hence most attine research focused first on antibiotics. Finding that most microbes in the biofilms of attine ants also inhibit *Escovopsis* is therefore no surprise, particularly because *Escovopsis* seems easy to suppress (*Escovopsis* can be suppressed by secretions of yeasts [Rodrigues et al. 2009], a group of microbes not known for innovative antibiotic activities). Recent research began to rectify the exclusive focus on *Escovopsis* suppression and began exploring additional sanitary functions [Haeder et al. 2009; Sen et al. 2009; Barke et al. 2010; Schoenian et al. 2011; Mattoso et al. 2012], but a number of other functions (Table 1, main article) remain untested, as well as the possibility that the integumental biofilms may perhaps be detrimental to the ants under some conditions (e.g., detrimental in specific ant populations, in specific ant castes, during specific seasons in temperate attine ant species, etc). Whereas it is easy to document antibiotic activity *in vitro* for most microbes associated with a host, it requires special chemical analyses to establish that any such microbe actually contributes beneficially to host fitness *in vivo* through antibiotic secretion [Schoenian et al. 2011]. Future research will need to adhere more stringently to established principles used to document adaptive design and adaptive association of a putatively beneficial microbe.

Review bias: Publications on ant-microbe research were often not peer-reviewed by microbiologists, but by entomologists with no training in microbiology. For example, because of an oversight, the microbial methods for isolating *Pseudonocardia* and for antibiotic testing were not published until 2005 [Cafaro & Currie 2005], which greatly delayed replication of the original experiments by other research groups. Review of the earliest studies by microbiologists undoubtedly would have prevented the omission of key microbial methods from publications, and most likely also the premature conclusion of antibiotic specificity and ant-*Pseudonocardia*-*Escovopsis* co-evolution.

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