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A mutualistic microbiome

How do fungus-growing ants select their antibiotic-producing bacteria?

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We recently published a paper titled "A mixed community of actinomycetes produce multiple antibiotics for the fungus farming ant *Acromyrmex octospinosus*" showing that attine ants use multidrug therapy to maintain their fungal cultivars. This paper tested two theories that have been put forward to explain how attine ants establish mutualism with actinomycete symbionts: environmental acquisition versus co-evolution. We found good evidence for environmental acquisition, in agreement with other recent studies. We also found evidence that supports (but does not prove) co-evolution. Here we place the environmental acquisition and co-evolution arguments within the framework of general mutualism theory and discuss how this system provides insights into the mechanisms that assemble microbiomes. We conclude by discussing future directions for research into the attine ant-actinomycete mutualism.

Insect fungiculture has been best studied in the attine ants, whose common ancestor is estimated to have evolved agriculture around 50 million years ago. The cultivated fungus lives in a mutualistic symbiosis with the ants and is vertically transmitted by daughter queens.[1] Attine ants also engage in a mutualism with actinomycete bacteria that produce antibiotics for use as weedkillers in the fungal garden, to prevent it being overgrown by other microbes.[2-4] The relationship between actinomycetes, fungal cultivar and attine ant has been most intensively studied in the higher attines or leaf-cutting ants (genera Atta and Acromyrmex), which harvest fresh vegetation to feed their fungal cultivar, *Leucoagaricus gongylophorus*.[1] Pathogens of the fungal garden, most notably fungi of the genus Escovopsis, if left unchecked, can destroy a fungal garden within weeks.[5,6]

Two main theories have been put forward to explain the evolution of mutualism between attine ants and their antibiotic-producing actinomycetes.[7-9] The first posits long-term co-evolution between attine ants and actinomycete bacteria in the genus Pseudonocardia, in which the latter evolves in an arms-race fashion to produce compounds that target the specialized pathogen Escovopsis, so as not to inhibit the growth of the fungal cultivar.[9] The second theory suggests that attine ants take up and cultivate multiple antifungal-producing actinomycete bacteria from the soil environment. The latter theory is consistent with the recent documentation of multiple actinomycete genera, including antibiotic-producing Streptomyces and Amycalotopsis, on leaf-cutting ants,[4] whereas the former theory considers these to be environmental contaminants.

In our paper, we argue that these possibilities are not mutually exclusive.[10] We isolated a strain of Pseudonocardia and a strain of Streptomyces from a single colony of *Acromyrmex octospinosus* and showed that the two strains make different antifungal compounds, which is consistent with the ants using multi-drug therapy against their fungal pathogens. The Pseudonocardia that we isolated

makes an antifungal that has not been observed before and this is suggestive of, but not conclusive evidence for, co-evolution between Acromyrmex ants and Pseudonocardia bacteria.[10]

However, Pseudonocardia have not been as intensively investigated for their antibiotic-producing capability as other genera (e.g., Streptomyces), so it will be necessary to analyse multiple Pseudonocardia symbionts from other attine taxa and from the soil to see just how unusual this compound is. Notably, the antifungal we identified, nystatin P1, belongs to the polyene family, and these compounds are effective against a range of different fungi. Indeed, nystatin P1 is active against both the specialist attine nest pathogen *Escovopsis weberi* and against the human pathogen *Candida albicans*.[10] This finding is consistent with another recent study reporting that the antifungal compounds produced by actinomycetes living on attines have generalised antifungal activity and do not specifically target Escovopsis.[4]

We also presented evidence that, in agreement with previous studies, strongly suggests that *A. octospinosus* take up and host antifungal-producing actinomycetes from the soil, most notably Streptomyces species.[4,10,11] In fact this makes sense, because even if particular Pseudonocardia strains have co-evolved with the ants, they must also originally have been acquired from the environment. We isolated a candicidin-producing Streptomyces species that has been isolated previously from geographically and phylogenetically distinct species of Acromyrmex.[3] Repeated, independent isolations suggest that this Streptomyces species is a regular mutualist, but the fact that it produces a well-known antifungal that is widespread in non-ant-associated isolates of Streptomyces also suggests that the ants acquired this species from the environment recently.[3,10,12]

Despite recent progress into this complex mutualism, fundamental questions still remain. (1) How do ants select useful bacteria from the diverse microbial soil community and (2) how do mutualistic bacteria outcompete other bacteria to maintain their symbiosis with their ant hosts?

These questions are the focus of two theories of mutualism, known as Partner Fidelity Feedback and Partner Choice. Under Partner Fidelity Feedback (PFF), an exogenous force (e.g., co-dispersal) aligns the interests of two lineages and causes mutualistic behavior to evolve, because when the symbiont helps the host, the healthier host automatically feeds back benefits to the symbiont.[13] Thus, if Pseudonocardia is vertically transmitted, ant colonies hosting beneficial Pseudonocardia strains would be more fecund, and this would confer higher fitness on those Pseudonocardia strains. Therefore, under PFF, Pseudonocardia was recruited early in the evolution of attine ants and, because of co-dispersal, has evolved to become a mutualist. PFF theory is well supported[13] and is one reason why the co-evolution explanation of the ant-Pseudonocardia relationship has been accepted for so long.

In contrast, the environmental recruitment idea invokes the Partner Choice (PC) model. PC posits that hosts select currently beneficial symbionts out of a pool of all symbionts, to bring about mutualism.[15] The outstanding question in the attine-actinomycete symbiosis is how to maintain antibiotic efficacy, given that specialist pathogens should evolve resistance. Regular recruitment of multiple actinomycetes from the soil and the multiple antibiotics that they produce could help the ants slow down the evolution of resistance. If this is the case, how do ants avoid taking up useless or even harmful bacteria? Recent theory suggests that microeconomic 'screening' games can provide mechanisms for successful PC.[16] If a host can set up 'working conditions' that favor mutualists and disfavor parasites, only beneficial symbionts will evolve to colonize such hosts. Thus, even the environmental recruitment hypothesis could also require evolution, but of a different kind to PFF.

A potential example of screening in the attine-actinomycete symbiosis is suggested by Sen et al.[4] who noted that antibiotic production may be selected for if it confers competitive superiority for the attine-ant niche. Thus, antibiotic activity against garden pathogens could be thought of as a by-product. A screening interpretation would further suggest that the ant host has evolved to promote the right kind of bacterial competition by providing the right mix of resources to fight over. Although the antifungals identified in the *A. octospinosus* system do not have antibacterial activity, each actinomycete species typically produces multiple antibiotics and it will be interesting to assay antibacterial activity in ant-associated actinomycetes. Moreover, since soil actinomycetes are known to share antibiotic resistance genes via horizontal gene transfer,[17] it is feasible that all the actinomycetes found in attine ant nests are resistant to all the antimicrobials being produced, thus allowing them to co-colonise the ant niche.

In summary, there is good evidence for environmental recruitment of antibiotic-producing actinomycetes by attine ants[4,10,11] and for the idea that actinomycete-produced antibiotics are important for helping the right bacteria to colonize and establish mutualism with attine ants. Recent studies have shown that the co-evolved Pseudonocardia mutualists are not phylogenetically very distinct from environmental isolates and that the antifungals produced by these bacteria have generalised activity rather than specifically targeting Escovopsis.[4,8,10] Careful experimental work will be required to determine which antibiotics are being produced in situ in the ant nests and whether the Pseudonocardia mutualists really are producing novel compounds. Currently, the draft genome sequence we deposited as part of our previous study[10] is the only Pseudonocardia genome sequence in the database. However, more free-living and mutualist genomes should soon be available for comparative analyses and we take this opportunity to announce a second draft genome of an attine ant-associated Pseudonocardia species described by Barke et al.[10] (Accession = AEGE00000000). We hope that this will stimulate other research groups to do the same.

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