

Bacterial Protection of Beetle-Fungus Mutualism

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The pervasiveness of beneficial associations between symbiotic microbes and plants and animals in every ecosystem illustrates how the acquisition of a microbe's physiological capacity confers substantial fitness benefits to hosts (1). However, dependence on mutualistic microbes becomes a liability if antagonistic microbes attack or outcompete beneficial ones (2). Therefore, mechanisms to preserve beneficial microbes must be a widespread, although poorly understood, component of host-microbe mutualisms. We show that a beetle uses a bacterium to protect its fungal food source from a competitor fungus.

Southern pine beetles, *Dendroctonus frontalis*, engage in a beneficial symbiosis with the fungus *Entomocorticium* sp. A, which provides nourishment for their developing larvae. Adult beetles carry *Entomocorticium* sp. A in a specialized storage compartment called a mycangium (Fig. 1A), excavate ovipositional galleries within the inner bark and phloem of host pine trees, and inoculate these galleries with *Entomocorticium* sp. A (3, 4). The success of the *D. frontalis*-*Entomocorticium* sp. A mutualism is challenged by an antagonistic fungus, *Ophiostoma minus*, which can outcompete *Entomocorticium* sp. A and thereby disrupt beetle larval development (3, 4). Our results indicate that successful maintenance of the *D. frontalis*-*Entomocorticium* sp. A mutualism is likely mediated by an actinomycetous bacterium that produces antibiotics that selectively inhibit *O. minus*.

The presence of previously unknown actinomycetes within the *D. frontalis*-*Entomocorticium* sp. A mutualism was established by scanning electron microscopy (SEM) and enrichment culture isolations (5). SEM revealed unexpected and profuse growth of actinomycetes within the galleries of *D. frontalis*, as well as inside the mycangia (Fig. 1B and fig. S1A). Isolations from 110 beetle individuals yielded 846 colony-forming units (CFUs) of actinomycetes, including at least one CFU from each of 92 individuals. Out of 164 actinomycete CFUs selected to be transferred to pure culture, 99 isolates had a red morphotype, whereas 65 isolates had a white morphotype. DNA sequence analyses confirmed the visual morphotype distinction, and within each of the two morphotypes there was complete 16S rDNA sequence identity. The two morphotypes form a monophyletic clade closely related to *Streptomyces ther-*

mosacchari. Furthermore, we also isolated the same red morphotype from 5 of 10 mycangia sampled.

We explored the potential role of the actinomycetes in mediating the *D. frontalis* fungal community by using symbiont pairing bioassays and chemical analyses. The bioassays, which crossed all possible combinations of the two actinomycete morphotypes with *Entomocorticium* sp. A and *O. minus*, revealed that isolates of the red morphotype produced a diffusible activity that inhibits the beetle's antagonistic fungus, *O. minus*, but only slightly affects the beneficial fungus, *Entomocorticium* sp. A (Fig. 1C and fig. S1, B and C). Extensive chemical and spectral analyses on strains of the red morphotype revealed the antifungal molecule responsible for selective inhibition to be a polyene peroxide, which we named mycangimycin. Mycangimycin (C₂₀H₂₄O₄), which

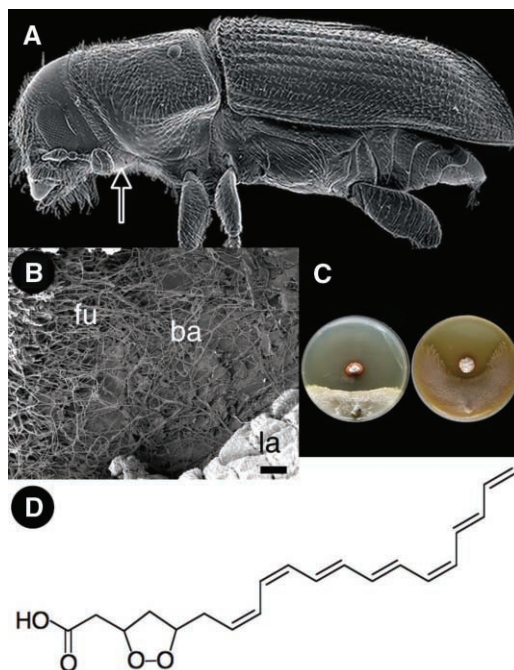


Fig. 1. (A) SEM micrograph of adult *D. frontalis* showing the location of a mycangium (arrow), which is used to transport *Entomocorticium* sp. A. (B) SEM micrograph from the *D. frontalis* gallery showing the actinomycetous bacterium (ba), fungus (fu), and beetle larva (la). (C) Representative examples of pairwise bioassay challenges illustrating inhibition of the fungal antagonist, *O. minus* (left), by a *D. frontalis* symbiotic actinomycete (strain SPB074). In contrast, the southern pine beetle's fungal mutualist, *Entomocorticium* sp. A, is relatively resistant (right) (see SOM for more details). (D) The structure of mycangimycin contains a seven-conjugated double bond chain and a five-membered endoperoxide ring.

has not been previously reported, is a linear 20-carbon carboxylic acid with an endoperoxide linking C-3 and C-5 to form a 1,2-dioxolane and a conjugated *cis, cis, trans, trans, cis, trans*-heptaene spanning C-7 to C-20 (Fig. 1D). Liquid culture antifungal assays using purified mycangimycin showed *O. minus* to be almost 20 times more susceptible [minimal inhibitory concentration (MIC) = 1.0 μM] than *Entomocorticium* sp. A (MIC = 19.0 μM) (fig. S1D). The identification of an actinomycete that is localized in the mycangium and galleries, which produces an antibiotic that selectively suppresses the antagonistic fungus, *O. minus*, indicates that *D. frontalis* engages in an additional mutualism with bacteria to regulate the *Entomocorticium* sp. A-*O. minus* fungal community. Because other bark-beetle species also depend on successfully maintaining beneficial fungi, tripartite beetle-fungus-bacterium mutualisms may be widespread.

Our study parallels earlier work on fungus-farming ants, which use actinomycetes to help protect their fungal gardens from pathogens (6). Taken together, these findings suggest that the use of antibiotic-producing actinomycetes may be a common method for maintaining beneficial microbes. Indeed, considering the importance of pathogens as a driving force in the evolution of all hosts, the benefit of such associations may extend to helping protect plants and animals from pathogens to which they themselves are susceptible (7, 8). If, as seems likely, these associations are widespread, targeting them could be an effective strategy for locating novel biologically active natural products.

References and Notes

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Supporting Online Material

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Materials and Methods

Fig. S1

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