Supporting Online Material for

**Bacterial Protection of Beetle-Fungus Mutualism**
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Materials and Methods

Southern Pine Beetle (SPB) Collection. Sections of loblolly pine (Pinus taeda L.) logs were collected from SPB infestations located within the Homochitto National Forest, near Meadville, MS, USA. Logs were placed in emergence containers in a climate controlled insectary at the USDA Forest Service, Southern Research Station laboratories in Pineville, LA, U.S.A. As beetles emerged they fell into sterile refrigerated collection cups. Only live, moving beetles were used in this study.

Southern Pine Beetle Symbiosis. SPB reproductive adults bore holes through a tree’s outer bark to create ovipositional galleries within the inner bark and phloem (S1, S2). These galleries serve as the nursery for larvae. During the process of gallery construction, female SPBs inoculate the phloem and xylem of the tree with their mutualistic fungi Entomocorticium sp. A (S1, S2). The fungus colonizes the uninfected phloem within the newly constructed galleries and serves as an important food source for the developing larvae. Additional fungal symbionts occur within this insect-fungal community. One additional mutualistic fungus, Ceratocystiopsis ranaculosus, functions in much the same manner as Entomocorticium sp., although it is less beneficial to the beetle (3). A third symbiont, Ophiostoma minus, has a context-dependent relationship with the SPB: it can out-compete the SPB’s beneficial fungus for uncolonized substrate and thereby disrupt SPB larval development; it can also assist attacking adults in killing healthy trees (3, 4, S2). Both C. ranaculosus and O. minus engage in their own mutualism with tarsenemid mites, which ride from tree to tree on the exoskeleton of SPBs. The mites feed on both these fungi, and, in exchange, the fungi are vectored to new host trees (S2). Thus the SPB system includes at least five symbionts: one mutualistic pair – SPB and its beneficial fungus – a second mutualistic pair –
O. minus and the tarsonemid mites – with a net antagonistic relationship to the first pair, and the host tree in the middle.

In our study, we focused on the interactions between actinomycetes and Entomocorticium sp. A. and O. minus because the ecological roles of these fungal symbionts have been previously established. This allowed us to test the a priori prediction that if SPBs engage in a mutualistic association with actinomycetes (paralleling the fungus-growing ant system) the bacterial symbionts should inhibit O. minus, the fungus antagonistic to the beetles’ larvae, but not Entomocorticium sp. A, the fungus that benefits SPBs. Because the ecological role of C. ranaculosus is not clearly resolved, making a priori predictions of how it interacts with potential bacterial symbionts was not possible.

**Scanning electron microscopy (SEM).** To visualize potential actinomycetes associated with SPBs, we used 50 adult female SPB beetles and 20 beetle galleries, which were removed with sterile razor blades. All specimens were fixed in half-strength Karnovsky’s fixative (S3). Specimens were prepared for SEM by following previously published methods (S4). All the specimens were examined with a JSM-6500F scanning electron microscope (JEOL).

**Microbial Isolations.** Bacterial isolations were conducted on 110 female SPB adults following previously published methods (S5). In addition, isolations of actinomycetes present within the mycangia of SPB were conducted from 10 live beetles. The mycangia were dissected, surface sterilized, and particle plated on chitin media. Fungal symbionts were isolated from emerging SPBs by following previously published methods (S2).

**Bioassays.** Bioassay challenges were conducted between actinomycete isolates and the main fungi associated with the SPB system (Entomocorticium sp. A and Ophiostoma minus). Forty strains of each of the two morphotypes of actinomycetes found associated with SPB were used in the bioassays. Challenges between bacterial strains and each of the fungi where conducted on yeast malt extract agar and followed previously published methods (S5). Bioassays were run for six weeks or until fungal growth ceased.

**Isolation and structural determination of mycangimycin.** An isolate of the red morphotype obtained from the mycangium of a SPB (strain SPB074) was cultured in the medium YMEA (4 g yeast extract, 10 g malt extract, and 4 g glucose per 1 L) at 30 °C for 3 days. 20 mL of the YMEA culture was then inoculated to 200 mL of YPM (2 g yeast extract, 2 g peptone, and 4 g mannitol per 1 L). The YPM culture was incubated for about 15 h. The whole culture was extracted with ethyl acetate. The dry crude material was fractionated through a C18 column by eluting with water, methanol, and dichloromethane combinations. The methanol/dichloromethane 1:1 fraction was subsequently purified by partitioning with hexane to yield pure mycangimycin. 1H, 13C, and 2D NMR data were collected on a Varian Inova 600 MHz spectrometer. UV spectra were acquired in an Amersham Biosciences Ultraspec 5300 Pro spectrometer. A high-resolution mass spectrum was obtained using Waters Q-Tof Ultima with chemical ionization source. The structure of mycangimycin was determined by NMR, UV, and mass spectral data. The production of mycangimycin was confirmed in six of six isolates of the red morphotypes and by none of the white morphotypes (n=3).
Antifungal assay. *Ophiostoma minus* and *Entomocorticium* sp. A strains were cultivated in potato dextrose medium (Difco). The cell suspensions were transferred to the wells in 96-well plates and various concentrations of pure mycangimycin were applied. The plates were incubated for 24 h and alamarBlue (Soretec Ltd.) was added. Fluorescence was measured with excitation at 540 nm and emission at 590 nm by Wallac Vector 2 plate reader after 6 h.

Vouchers, sequences and phylogenetic analysis. We sequenced ~1445 base pairs of 16S rDNA from 33 strains of actinomycetes isolated from SPB (red=20 and white=13). Sequence analysis revealed 100% (1445/1445) sequence identity within morphotypes and 99.5% (1438/1445) between isolates of red and white morphotypes. The evolutionary relationship among the actinomycetes isolated from the SPB system was elucidated by phylogenetic analyses (*Scott et al.* unpublished data). Maximum parsimony (MP) and maximum likelihood analyses (ML) were performed in PAUP* 4.0 using heuristic searches (stepwise addition and tree bisection–reconnection branch swapping). All characters were treated as unordered, and gaps as missing data. Bootstrap support for MP was calculated for internal branches after 1000 pseudoreplicates. The model of sequence evolution was estimated using ModelTest 3.7.1, indicating the general time reversible model (GTR+Γ+G) under a gamma distribution for the among-site rate variation (base frequencies = (A:0.2137 C:0.2452 G:0.3445 T:0.1996); rate matrix = (AC:0.5306 AG:1.8143 AT:0.9797 CG:0.7552 CT:4.3220 GT:1.0000); shape parameter for gamma distribution=0.5063; number of estimated parameters for discrete gamma approximation=10; proportion of invariant sites=0.3256). These analyses revealed that the actinomycetes associated with SPB form a monophyletic clade, most closely related to *Streptomyces thermosacchari*.

The 16S rDNA sequences of SPB bacterial strains are available at the NCBI’s GenBank database under the accession numbers EU798707-EU798708. Bacterial strain SPB074 is on deposit at ATCC® under the Patent Deposit Designation number PTA-8607. Fungal strains are deposited in the Forest and Agricultural Biotechnology Institute Culture Collection (M. J. Wingfield, University of Pretoria, South Africa).

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Fig S1.

(A) SEM micrograph of SPB gallery in loblolly pine, showing a nearly complete view of a SPB larva (la), and growth of fungal (fu) and actinomycetous (ba) symbionts (top). Micrograph from the same SPB larval gallery, illustrating filamentous bacteria (solid arrows) growing interwoven with the mutualistic fungi (open arrow) (bottom left). Micrograph image of a SPB mycangium, with arrows indicating actinomycete growth within the gland cells surrounding the mycangium in the prothorax (bottom right). Scale bars, 200 μm, 10 μm, 1 μm, respectively. (B) Representative examples of pairwise bioassay challenges between the two actinomycete strains (red morphotype = ‘StrainR’ (top) and white morphotype = ‘StrainW’ (bottom)) and the two fungi *Ophiostoma minus* (beetle antagonist) or *Entomocorticium* sp. A (beetle mutualist). The actinomycete is in the middle, while the fungus is inoculated at the edge of the Petri plate. Zones of inhibition (zoi, space between the bacterium and fungus) indicate inhibition of fungus by antibiotic produced by the bacterium (e.g., upper right image). (C) Summary of 160 bioassay challenges, indicating the frequency of different actinomycete strains of each morphotype displaying different degrees of ability to inhibit the two fungi. Black indicates strong inhibition (zoi > 2.5 cm) of the fungus by the bacterium, grey indicates mild inhibition (zoi 0.5-2.5 cm), and light grey indicates little or no inhibition (zoi < 0.5 cm). (D) The susceptibility dose response curves plotting log concentration mycangimycin by fungal density of *O. minus* and *Entomocorticium* sp. A, illustrating selective inhibition of the beetle’s antagonistic fungus, *O. minus*, by mycangimycin.