

BLACK YEAST SYMBIONTS COMPROMISE THE EFFICIENCY OF ANTIBIOTIC DEFENSES IN FUNGUS-GROWING ANTS

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Abstract. Multiplayer symbioses are common in nature, but our understanding of the ecological dynamics occurring in complex symbioses is limited. The tripartite mutualism between fungus-growing ants, their fungal cultivars, and antibiotic-producing bacteria exemplifies symbiotic complexity. Here we reveal how black yeasts, newly described symbionts of the ant–microbe system, compromise the efficiency of bacteria-derived antibiotic defense in fungus-growing ants. We found that symbiotic black yeasts acquire nutrients from the ants' bacterial mutualist, and suppress bacterial growth. Experimental manipulation of ant colonies and their symbionts shows that ants infected with black yeasts are significantly less effective at defending their fungus garden from *Escovopsis*, a prevalent and specialized pathogen. The reduction of mutualistic bacterial biomass on ants, likely caused by black yeast symbionts, apparently reduces the quantity of antibiotics available to inhibit the garden pathogen. Success of the ant–fungal mutualism is directly dependent on fungus garden health. Thus our finding that black yeasts compromise the ants' ability to deal with the garden parasite indicates that it is an integral component of the symbiosis. This is further evidence that a full understanding of symbiotic associations requires examining the direct and indirect interactions of symbionts in their ecological community context.

Key words: Apterostigma; Attini; bacteria; ecological community dynamics; Escovopsis; interspecific interaction; symbiosis; yeast.

INTRODUCTION

Symbiotic associations shape the biology of all organisms and have been fundamental in many of the major evolutionary transitions toward greater organismal complexity and diversity (Ehrlich and Raven 1964, Pirozynski and Malloch 1975, Margulis and Fester 1991, Thompson 1994, Van der Heijden et al. 1998, Lutzoni et al. 2001). For example, microbial symbionts mediated the origin of the eukaryotic cell, facilitated the colonization of land by plants, and aid digestion in animals. Traditionally, many symbioses have been studied in a binary framework, which allowed for more practical empirical studies and more tractable theoretical models. However, it is now evident that many host-microbe pairs are part of a more complex network of beneficial and antagonistic symbionts (Bronstein and Barbosa 2002, Moran et al. 2005). Unlike the direct effects pathogens have on their hosts, or mutualists have on one another, the majority of interactions that occur among community members are indirect, i.e., those that require an intermediate species to be transmitted (Wootton 1994). Indirect interactions can influence both the outcome and the cost–benefit ratio of symbiotic interactions (Letourneau 1990, Currie et al. 1999a, b, Gastreich 1999).

Furthermore, selection on traits that are important to maintaining successful symbioses have the potential to be influenced indirectly by diffuse symbionts as do the coevolutionary dynamics of codependent organisms.

Ants in the tribe Attini have an ancient obligate association with fungi that they cultivate for food. The nutritional benefit provided to ants by their mutualistic fungus is a prime target for exploitation by other organisms. As such, fungus-growing ants have evolved various mechanisms to defend their fungus garden, including hygienic behaviors to rid the garden of foreign microbes, and management of hazardous garden waste (Weber 1972, Currie et al. 1999b, Bot et al. 2001, Currie and Stuart 2001, Hart and Ratnieks 2002, Fernandez-Marin et al. 2006). Despite these defensive mechanisms, the ant–fungal mutualism is parasitized by a lineage of specialized pathogens in the genus *Escovopsis* (Currie et al. 1999a, Currie 2001, Reynolds and Currie 2004). To help protect their fungal crops from the specialized pathogen, the ants engage in a second mutualism with bacteria in the genus *Pseudonocardia* (actinomycete), which produce antibiotics that specifically inhibit *Escovopsis* (Currie et al. 1999b). The mutualistic bacteria typically occur on, or in, specialized cuticular structures that are connected to unique exocrine glands, which apparently produce nutrients to support bacterial growth (Currie et al. 2006). Thus, each member of the fungus-growing ant community is directly and indirectly

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influenced by one another in both positive and negative ways.

While isolating the bacterial mutualists from species of *Apterostigma*, a phylogenetically basal genus of fungus-growing ants, we frequently obtained colonies of black yeasts (closely related to fungi in the genus *Phialophora*; Ascomycota). This monophyletic group of black yeasts are prevalent within and among ant colonies, are symbiotically associated with fungus-growers from across the phylogenetic and geographic distributions of the mutualism, and occupy a specific niche on the bodies of fungus-growing ants (Little and Currie 2007). Here we link the multiple interactions occurring in the ecological community of the fungus-growing ant-microbe symbiosis by exploring and integrating the impacts of direct and indirect interactions among symbionts. Specifically we test the hypothesis that black yeasts exploit the ant-bacterial mutualism by consuming the ants' mutualistic bacteria. Black yeast interactions with *Pseudonocardia* are investigated through bioassays. To investigate the impact black yeasts have on ant-microbe mutualists experiencing stress caused by garden infection, we experimentally manipulate ant colonies. In addition, to further understand the results of this experiment we perform a series of bioassay challenges between microbial symbionts in vitro. We discuss mechanistic details of the complex ecological role the black yeast symbiont plays in the ant-microbe symbiosis, and suggest that the multiple indirect interactions of fungus-growing ant symbionts have helped structure the ecological community over evolutionary time.

MATERIALS AND METHODS

Study organisms

Apterostigma spp. are common throughout much of Central and South America. Ants house their fungus gardens under rocks or decaying logs, between the stilts of palms, or in moist soil on the vertical planes of ravine banks (Weber 1972). They manure their fungi with decaying vegetation and insect frass. The colonies of *Apterostigma pilosum* used in this study were collected between 2002 and 2006 in the Republic of Panama (Canal Zone, Darien Province, Bocas del Toro Province), and were maintained in the laboratory in dual plastic chambers placed on islands in mineral oil, to prevent horizontal transfer of microbes among colonies. Colonies were watered and fed a mixture of oats, corn meal, tea leaves, and oak catkins once a week. Voucher samples of ants from each colony were collected and stored at -80°C in ethanol, and deposited at the Smithsonian Institute.

Isolation of microbes

Black yeasts and actinomycetes were isolated from *A. pilosum* following a protocol used to isolate actinomycetes from fungus-growing ants (Cafaro and Currie 2005). Ants were scraped using a sterile metal probe and

the material was spread on chitin agar. Plates were incubated at room temperature for four weeks. Individual colony forming units (CFUs) of black yeast were then transferred to, and maintained on potato dextrose agar (PDA) (Difco, Sparks, Maryland, USA). Individual CFUs of *Pseudonocardia*, the ants' bacterial mutualist, were transferred to yeast malt extract agar (YMEA).

Black yeast growth on water and Pseudonocardia agar

To determine whether the black yeast is capable of using the ant's bacterial mutualist as a nutrient source, 0.2-mm³ plugs of the fungus were placed on two types of media: (1) *Pseudonocardia* agar, which is water agar containing 2.0 g/L of *Apterostigma*-associated *Pseudonocardia* tissue from pure culture, and (2) water agar (15 g agar/L H₂O). Cultures were incubated at room temperature for four weeks, and surface area growth was measured (mean of three diameter measurements) and compared using a two-sample *t* test.

Infection experiment

To determine whether exploitation of the ant-bacterial mutualism by black yeast disrupts the ants' ability to defend their fungus garden, we conducted experiments crossing the presence/absence of black yeasts with the presence/absence of *Escovopsis* using subcolonies of *A. pilosum*. Subcolonies of *A. pilosum*, composed of 50 mg of fungus garden and six worker ants, were set up in 60-mm plastic Petri plates with moist cotton along the outer edge. Each subcolony was fed a mixture of oak catkins and tea leaves daily, and subcolonies were deemed healthy once ants started incorporating substrate into the fungus garden. Each subcolony was randomly assigned to one of four treatments: (1) black yeast removed and no infection, (2) black yeast removed and infection with *Escovopsis*, (3) black yeast not removed and no infection, and (4) black yeast not removed and infection with *Escovopsis*.

To remove black yeasts from ants, workers were removed from their subcolony, placed in a 4-mL glass vial, and exposed to 1.0% Amphotericin B (AmpB) three times during a 24-hour period (approximately every eight hours). AmpB is a polyene antifungal that preferentially binds to ergosterols, thus disrupting osmotic integrity of the fungal cell membrane (Terrell and Hughes 1992). This method inhibited black yeasts to the point where they were undetectable via culturing methods for at least one month, but did not measurably affect actinomycete growth on ants ($n = 25$ replicates). Ants were given 30 minutes to groom themselves after exposure to antibiotics, and then returned to their subcolonies. To determine if AmpB adversely affects the other symbionts in the fungus-growing ant microbe symbiosis, fungal cultivar, *Escovopsis*, and *Pseudonocardia* isolated from *Apterostigma* were all incubated on PDA (fungal) and YMEA (bacterial) containing 1.0% AmpB overlays. Unlike the black yeast symbionts,

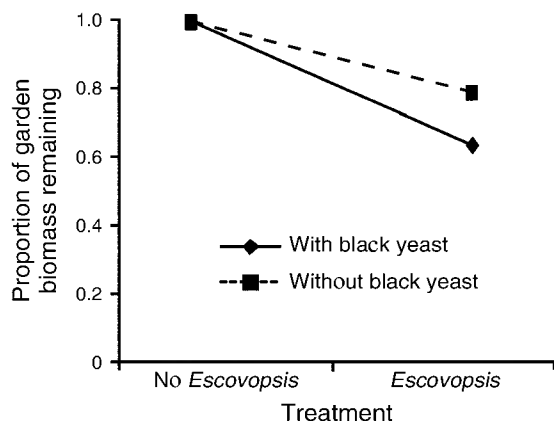


FIG. 1. Two-way interaction graph illustrating the effects of black yeast and *Escovopsis* infections in *Apterostigma pilosum* subcolonies. Ants infected with black yeasts (solid line) are significantly worse at defending their garden from *Escovopsis* infection than are uninfected ants (dashed line). Nonparallel lines indicate that the interaction of the two treatments is significant. The standard error for each data point is less than 0.01; thus bars are not shown.

which are unable to grow on YMEA with a 1% AmpB overlay, the growth rate of fungal cultivar, *Pseudonocardia*, and *Escovopsis* symbionts were not affected by the presence of the antibiotic (t test, $P > 0.1$, $df = 13$).

Prior to being used in the experiment, ant colonies were tested to confirm an absence of detectable *Escovopsis* infection (Currie 2001). The strain of *Escovopsis* (brown morphotype; see Gerardo et al. [2006]) used for infection was isolated in Gamboa, Panama from an *A. pilosum* colony. The isolate was grown on PDA with 1000 iu/mL of penicillin-streptomycin (MP Biomedicals, Aurora, Ohio, USA). Spores were added to ddH₂O (dd = double-distilled) with Tween 20 (5×10^{-5} mL/L) (Fisher Scientific, Pittsburgh, Pennsylvania, USA) to evenly disperse spores in solution. Each subcolony received 50 μ L of solution (~6000 spores per subcolony) via mist inoculation. Subcolonies that did not receive the *Escovopsis* infection treatment were sprayed with an equal amount of ddH₂O/Tween 20 solution.

Changes in fungus garden biomass were measured as a fitness indicator. Garden biomass was measured 72 hours after treatment, and proportions of garden biomass (relative to original amount) were subjected to two-way ANOVA in Minitab (Minitab Version 14 for Windows, Minitab, Inc., State College, Pennsylvania, USA). Graphical results (Fig. 1) show the two-way interaction between the AmpB and *Escovopsis* "infection" treatments.

Bioassay challenges

To determine the cause of black yeast associated garden loss found in infection experiments we performed a series of co-culture bioassay challenges in which we incubated different pairs of symbionts side by side on

nutrient medium in Petri dishes (Fig. 2a), and then compared their growth in the combined cultures with their growth in pure culture (Fig. 2b). Bioassay challenges between the black yeast and *Pseudonocardia* were conducted in Petri plate dishes containing YMEA, which allows for good growth of both symbionts, while pairings of black yeast with the fungal cultivar and *Escovopsis* were completed on PDA, which supports fungal growth. Black yeast hyphal material (2 mm^2) was inoculated onto each plate 1.5 cm away from a point inoculation of a *Pseudonocardia*, *Escovopsis*, or fungal cultivar strain. The growth of each CFU (colony-forming unit) was measured 10, 20, and 30 days after inoculation by taking three diameter measurements of the CFUs (which grow in a circular pattern), and calculating the surface area of the CFU from the mean diameter. Surface areas of each CFU were then subjected to two-sample t tests in Minitab to identify significant changes in growth. Solid medium was used for these challenges because in liquid, the yeast and actinomycetes get intertwined, making it difficult to obtain an accurate measurement of each strain's biomass.

To determine whether black yeasts suppress antibiotic production by *Pseudonocardia*, separate bioassay challenges between the black yeast and *Pseudonocardia* were set up, as above, and after 10 days, *Escovopsis* was point-inoculated at the edge of the Petri plate. Subsequent growth of both *Pseudonocardia* and *Escovopsis* were measured five days later, and compared to growth on control plates (i.e., no black yeast) to identify differences in the bacterial-parasite interaction. Specifically we quantified the difference in *Pseudonocardia*'s ability to inhibit *Escovopsis* by measuring the zone of inhibition between the two microbes on control and black yeast infected plates.

RESULTS

Black yeast growth on water and *Pseudonocardia* agar

Black yeast symbiont growth on water agar was minimal (mean surface area growth = $316.16 \pm 120.9 \text{ mm}^2$ [mean \pm SD]). However, incubation on water agar containing biomass of *Pseudonocardia* revealed significantly greater growth of black yeast, (two-sample t test, $df = 65$, $t = -3.54$, $P = 0.0007$; mean surface area growth $473.23 \pm 224.46 \text{ mm}^2$). This result indicates that the black yeast is capable of utilizing *Pseudonocardia*-derived nutrients.

Infection experiment

No difference in ant survivorship in the subcolonies of *A. pilosum* was detected in our infection experiment (t test, $df = 9$, $t = 0.91$, $P = 0.3865$), nor were we able to detect any changes in fungal garden biomass when black yeasts were present or absent from ants tending the garden (t test, $df = 9$, $t = 1.34$, $P = 0.2131$). Additionally, ants do not exhibit any observable behavioral aversion to black yeast tissue placed in their colonies (A. Little,

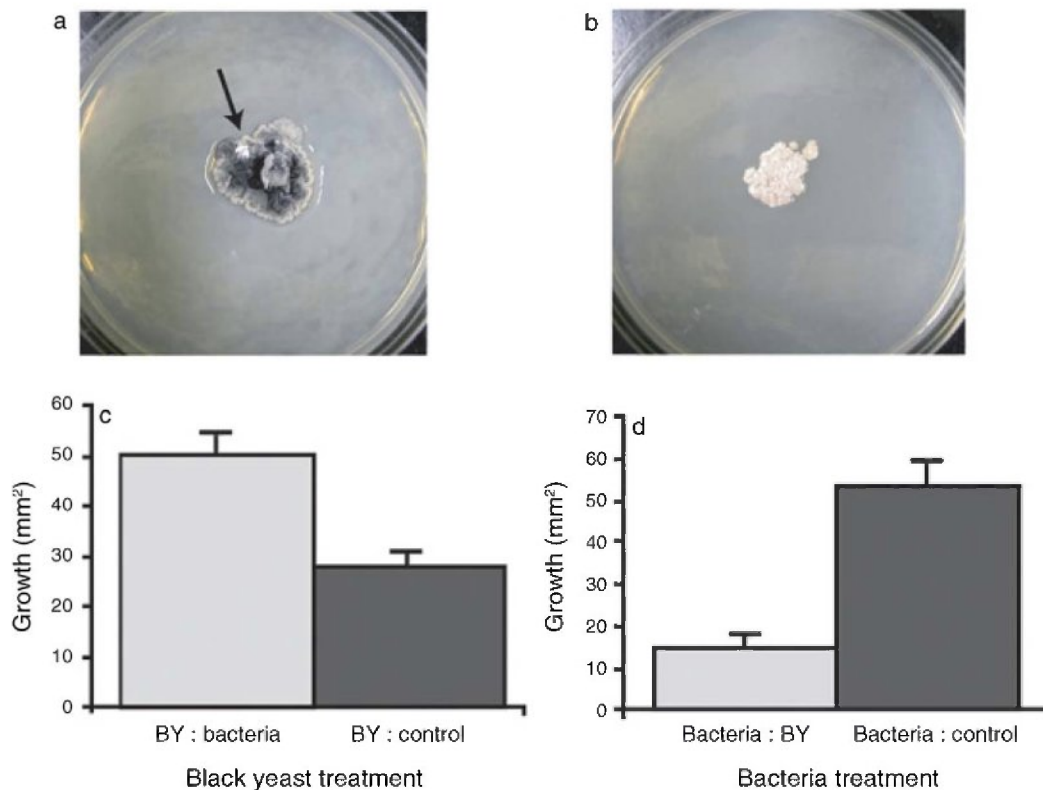


FIG. 2. Bioassay challenges used to investigate black yeast–*Pseudonocardia* interactions. Photos of (a) bioassay challenges between black yeasts and *Pseudonocardia* (arrow points to white *Pseudonocardia* growth), and (b) growth of *Pseudonocardia* in solitary culture. (c and d) Results from bioassay challenges indicating that *Phialophora* (black yeast) growth increases at the expense of *Pseudonocardia* (ants' bacterial mutualist) growth when allowed to grow side by side on yeast malt extract medium for 20 days at room temperature. (c) Bar chart showing black yeast (BY) growth in the presence of bacterial mutualists (left), and growth alone on nutrient medium (right). (d) Bar chart showing *Pseudonocardia* (bacterial mutualist) growth in the presence of black yeast isolated from *Apterostigma* (left), and growth alone on nutrient medium. Error bars represent + SD.

personal observation). As in previous experiments *Escovopsis* infection has a significant negative impact on fungus garden biomass (t test, $df = 9$, $t = -36.89$, $P < 0.0001$). Interestingly, through experimental manipulation of *A. pilosum* subcolonies we found that significantly more fungus garden biomass was lost in colonies treated with *Escovopsis* and tended by workers infected with black yeasts than in other treatments (ANOVA, $F_{1,43} = 163.31$, $P < 0.001$; Fig. 1). More specifically, when fungus-growing ant colonies are infected with the garden parasite *Escovopsis*, the presence of black yeasts reduces the ants' ability to suppress infection of their fungus garden.

Bioassay challenges

Pseudonocardia inhibition of *Escovopsis* *in vitro* was not altered by the presence of black yeast (t test, $df = 45$, $P = 0.6265$, $t = -0.49$). Results from paired bioassay challenges indicated that black yeasts do not significantly promote growth of *Escovopsis* (t test, $df = 45$, $P = 0.5648$, $t = 0.58$), nor does *Escovopsis* significantly alter black yeast growth (t test, $df = 45$, $t = -0.455$, $P = 0.6513$). In addition, co-culture experiments of black

yeast and the ants' cultivated fungi revealed no significant decrease in the growth of fungal cultivars or significant increase in growth of the black yeast (fungal cultivars t test, $df = 45$, $P = 0.4814$, $t = 0.71$; black yeast t test, $df = 45$, $P = 0.3472$, $t = 0.95$). Bioassay challenges between black yeast and *Pseudonocardia* (Fig. 2a, b) revealed that black yeast growth significantly increases in the presence of *Pseudonocardia* (t test, $P < 0.0001$, $t = -4.58$, $df = 54$; Fig. 2c). In contrast, the presence of black yeast resulted in a significant reduction in *Pseudonocardia* growth (t test, $P < 0.001$, $t = 7.18$, $df = 13$; Fig. 2d).

DISCUSSION

Our study exploring complex species interactions within the fungus-growing ant–microbe symbiosis reveals that the recently discovered black yeast symbionts exploit the ant–bacterial mutualisms. Infection experiments and behavioral observations suggest that black yeasts do not directly impact the health of either fungus-growing ants, or their cultivated fungal mutualists. However, the black yeast is able to derive nutrients directly from *Pseudonocardia*, the ants' antibiotic-

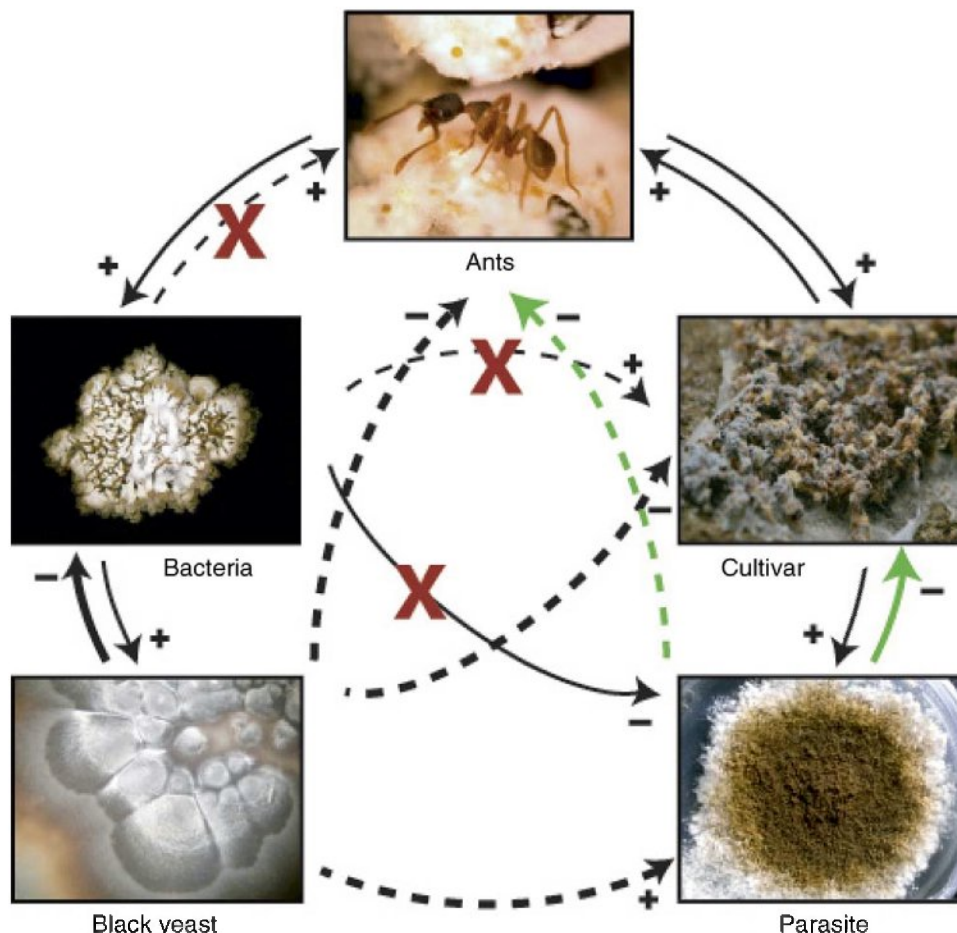


FIG. 3. Web diagram of the direct and indirect interactions of the attine ant-microbe symbiosis. The inclusion of indirect interactions illustrates how black yeast symbionts compromise the efficiency of antibiotic defenses of fungus-growing ants. Solid lines represent direct effects, which are the result of a physiological interaction between two species. Dashed lines represent indirect effects, which require the presence of an intermediary (species) to arise. The cost (-) and benefit (+) to each organism is indicated at the tip of the arrowhead. Bold, thick black lines indicate the direct (solid line) and indirect (dashed lines) effects black yeasts have on fungus-growing ant symbionts. Black yeasts directly inhibit growth of the ants' bacterial mutualist, which results in less efficient suppression of the garden parasite (indicated by a red X). This indirectly decreases the benefit bacteria provide ants and cultivars (also indicated by red X's). Consequently, black yeasts indirectly benefit the garden parasite by compromising antibiotic defenses, which increases the cost the parasite inflicts on ants and cultivar (indicated by green arrows).

producing mutualist, which increases the growth rate of the black yeast while slowing the growth of *Pseudonocardia*. The direct impact black yeast has on *Pseudonocardia* suggests it has the potential to indirectly impact the health of ants by disrupting their ability to inhibit the parasite *Escovopsis* via their antibiotic-producing bacteria. Indeed, experimental manipulation of ant colonies, crossing the presence/absence of black yeast with presence/absence of the garden parasite *Escovopsis*, revealed that subcolonies infected by black yeasts were significantly more impacted by *Escovopsis* infection (Fig. 1). Thus, our results indicate that both direct and indirect effects link multiple interactions among the fungus-growing ant-microbe symbionts and have likely been a significant factor structuring the ecological community over evolutionary time.

Our finding that the presence of black yeast symbionts on ants increases the virulence of *Escovopsis* could be the result of several mechanisms. Ants would be less adept at defending their fungus garden from *Escovopsis* if the black yeasts inhibit antibiotic production by the bacterial mutualists. However, we were unable to detect any significant difference in the *in vitro* ability of *Pseudonocardia* to inhibit *Escovopsis* in the presence or absence of the black yeast, indicating that ant-associated black yeasts do not inhibit antibiotic production by the *Pseudonocardia*. Another mechanism that might increase the virulence of *Escovopsis* is that black yeasts may directly stimulate its growth. Similarly, the decreases in fungus garden biomass observed during the fitness experiment could result if black yeasts inhibit or kill fungal cultivars. However, we did not find evidence that

black yeasts promote *Escovopsis* growth, or suppression or killing of fungal cultivars. Alternatively, if black yeasts are capable of significantly inhibiting the growth of the *Pseudonocardia* population on ants, the ants may be less able to defend their gardens from *Escovopsis*. Results from bioassay challenges showing that black yeast growth increases in the presence of the bacterial mutualist at a cost to *Pseudonocardia* growth support such a hypothesis. In addition to directly parasitizing *Pseudonocardia*, it is still possible that black yeasts also directly exploit the ant–bacterial mutualism by outcompeting the bacteria for nutrients produced by the ants, as both promotion of growth in the presence of the bacteria and the ability to inhibit bacterial growth, would increase the competitive ability of the black yeast within the ant–bacterial niche.

Understanding how interspecific interactions within communities drive changes in species abundance, distribution, and composition has been a common theme in ecology for over a century. However, studies clearly demonstrate that extracting pair-wise interactions from their community context to investigate ecological changes can be unrealistic and misleading (Sih et al. 1985, Bronstein and Barbosa 2002, Stanton 2003, this study). Most interactions among species are relatively weak and diffuse, but weak interactions can have strong effects when measured in the context of an ecological community (Paine 1992, Berlow 1999). This may be the case for the pair-wise interaction between black yeast and actinomycete symbionts. Although black yeasts significantly decrease the growth of actinomycetes in vitro, this interaction may be insignificant in vivo with respect to bacterial fitness since the association is so prevalent in nature. However, when this pair-wise interaction is examined within its community context the interaction becomes significant. The indirect effects black yeasts have on the fungus garden affect the fitness of the ant–cultivar–bacteria tripartite mutualism. Interestingly, the black yeast acts synergistically to benefit the fungal garden parasite by allowing it to infect the garden more severely. Synergism is not uncommon in pathogenesis. Herbivores increase the impact of many plant pathogens (Caesar 2003), UV-B increases the severity of several amphibian fungal pathogens (Kiesecker and Blaustein 1995) and viral–bacterial synergism causes excessive mortality during influenza outbreaks (Jones et al. 1983). Ants infected with black yeasts likely have fewer bacteria available to produce antibiotics that inhibit *Escovopsis* spores from infecting their fungal cultivar, thus the black yeast indirectly synergize *Escovopsis* infection. An interesting point to consider is whether the black yeast and *Escovopsis* are actually diffuse mutualists. Currie et al. (2003) showed that actinomycetes become more prevalent on the cuticle of ants during *Escovopsis* infection. The increased growth of *Pseudonocardia* stimulated by *Escovopsis*, could in turn benefit black yeasts, which derive nutrients from the bacteria. Studies monitoring

the abundance of *Pseudonocardia* and black yeasts on the ants' cuticle during different levels of infection would shed light on this hypothesis.

Understanding the antagonistic and beneficial interactions occurring in the ant–microbe symbiosis clearly requires an understanding of the contributions made by black yeast symbionts. Between 33% and 75% of fungus-growing ant colonies have detectable *Escovopsis* infections, which are lethal to fungus gardens if not adequately controlled by the ants (Currie 2001). Thus black yeasts, which decrease mutualistic bacterial biomass and indirectly increase the virulence of *Escovopsis*, have the potential to substantially impact the fitness of fungus-growing ants in nature. Assessing community dynamics by examining indirect interactions may be particularly informative in communities that are strongly shaped by pathogens and/or parasites. Indirect effects that are weak or diffuse when community members are healthy may become strong effectors when community members are stressed by antagonists such as parasites or pathogens. The finding that a newly discovered symbiont has such strong ecological impacts on a well-studied symbiosis, in combination with the recent recognition that many other symbiotic communities involve greater species complexity than previously recognized, highlights the need for future research to explore mutualistic and host–pathogen associations in a community context.

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