Prokaryote–eukaryote interactions identified by using Caenorhabditis elegans

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Prokaryote–eukaryote interactions are ubiquitous and have important medical and environmental significance. Despite this, a paucity of data exists on the mechanisms and pathogenic consequences of bacterial–fungal encounters within a living host. We used the nematode Caenorhabditis elegans as a substitute host to study the interactions between two ecologically related and clinically troublesome pathogens, the prokaryote, Acinetobacter baumannii, and the eukaryote, Candida albicans. After co-infecting C. elegans with these organisms, we observed that A. baumannii inhibits filamentation, a key virulence determinant of C. albicans. This antagonistic, cross-kingdom interaction led to attenuated virulence of C. albicans, as determined by improved nematode survival when infected with both pathogens. In vitro coinfection assays in planktonic and biofilm environments supported the inhibitory effects of A. baumannii toward C. albicans, further showing a predilection of A. baumannii for C. albicans filaments. Interestingly, we demonstrate a likely evolutionary defense by C. albicans against A. baumannii, whereby C. albicans inhibits A. baumannii growth once a quorum develops. This counteroffensive is at least partly mediated by the C. albicans quorum-sensing molecule farnesol. We used the C. elegans–A. baumannii–C. albicans coinfection model to screen an A. baumannii mutant library, leading to the identification of several mutants attenuated in their inhibitory activity toward C. albicans. These findings present an extension to the current paradigm of studying monomicrobial pathogenesis in C. elegans and by use of genetic manipulation, provides a whole-animal model system to investigate the complex dynamics of a polymicrobial infection.

A. baumannii | C. albicans | Acinetobacter | pathogenesis | biofilm

In nature, microorganisms exist within polymicrobial communities (1, 2), which abound with complex multispecies dynamics (3). These ecological interactions in general and prokaryote–eukaryote interactions in particular, are likely important for the evolution and maintenance of microbial virulence toward humans (4, 5). Moreover, humans are often co-infected or colonized with multiple pathogens (1, 6), whose interactions may determine the virulence potential of either organism. Despite the abundance of polymicrobial encounters within nature, there is a scarcity of in vivo models that explore the biological and pathological systems of interacting species. Significant challenges exist with reproducing polymicrobial interactions (6), and thus a facile, genetically tractable model system is desperately needed.

The soil-dwelling nematode Caenorhabditis elegans, has been successfully used as an alternative host in the study of host–pathogen interactions (7). Thus far, its use has been limited to the investigation of monomicrobial infections, including those caused by a wide range of bacteria and fungi (7). Importantly, microbial virulence determinants found to be relevant in pathogenesis toward C. elegans have also been found to be important in pathogenesis toward other hosts, including mammals (7). Recently, Candida albicans, the most common human fungal pathogen, with an attributable patient mortality reaching 40% in the face of invasive disease (8), was shown to cause a persistent lethal infection of the C. elegans intestinal tract (9). C. albicans infection leads to overwhelming intestinal proliferation and filamentation through the worm cuticle (9). The ability to form biofilm and undergo a morphological transition from yeast to a filamentous form, are critical virulence determinants of C. albicans toward mammals and C. elegans (9–13). Given the significance of this fungus to human health and its common cohabitation with other microbes, particularly bacteria (14, 15), we used C. elegans to identify and study interactions between C. albicans and various prokaryotic species.

This report shows that C. elegans can be effectively used to study the dynamics of a polymicrobial infection, more specifically that between a prokaryote and a eukaryote. We concentrated on the interaction between C. albicans and the emerging gram-negative pathogen, Acinetobacter baumannii. We describe an antagonistic relationship between these pathogens, whereby A. baumannii inhibits several key virulence determinants of C. albicans such as filamentation and biofilm formation. The observed A. baumannii–C. albicans interactions resulted in reduced C. albicans pathogenicity, as determined by reduced worm lethality when infected with both pathogens. However, illustrating the complexity of the interaction and a likely evolutionary defense process, C. albicans demonstrates growth-dependent antibacterial properties, which appear mediated by the quorum-sensing molecule farnesol. Finally, we describe the utility of using bacterial genetics and the C. elegans–A. baumannii–C. albicans model to identify potential underlying molecular mechanisms of the observed interaction. Our results extend the use of C. elegans in the study of polymicrobial pathogenesis and provide further evidence of the likely importance of cross-kingdom interactions.

Results

A. baumannii and Other Bacteria Inhibit Filamentation of C. albicans in a C. elegans Coinfection Model. When C. elegans glp-4; sek-1 nematodes are infected with C. albicans, and are exposed to a liquid environment, the majority of worms die with C. albicans filaments penetrating through the worm cuticle (9). C. albicans filamentation within C. elegans begins within 24 h of liquid-medium exposure and peaks by 72 h (data not shown). We used this C. elegans–C. albicans model to evaluate the interactions between a range of bacteria and C. albicans. Remarkably, when nematodes were infected sequentially with C. albicans followed by infection with the gram-negative bacterium A. baumannii or Pseudomonas aeruginosa, filamentation by C. albicans was significantly inhibited (Fig. L4). In contrast, filamentation was minimally affected by the nonpathogenic Esche-

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The observed *A. baumannii*–*C. albicans* interaction within *C. elegans* remained robust despite modifying the environment of the coinfection assay with factors that promote *C. albicans* filamentation. The *A. baumannii*-mediated inhibition of filamentation was not altered by bovine serum (at different concentrations up to 75%) (Sigma), filament-inducing spider medium (18), the quorum-sensing molecule tyrosin (Fluka) (4 μg/ml–200 μg/ml), which promotes filamentation (19), or by increased nutrients in the liquid medium for the assay [up to 80% brain–heart infusion (BHI)].

The degree of inhibition of *C. albicans* filamentation in *C. elegans* depended on the initial *A. baumannii* inoculum into the liquid medium of the assay, with measurable inhibition occurring with an inoculum as low as 10^2 CFU/ml (Fig. 1B). Washed, heat-killed *A. baumannii* cells at various densities (equivalent to 10^3–10^6 CFU/ml) caused no inhibition of *C. albicans* filamentation (Fig. 1B and data not shown), indicating the requirement for live cells or a secreted factor from *A. baumannii*. Confocal laser microscopy of the *A. baumannii*–*C. albicans* interaction within *C. elegans* showed the striking phenotypic differences between coinfection and *C. albicans* infection alone (Fig. 1 C–J). Notably, such differences were observed within 24 h after coinfection.

**A Secretory Factor from *A. baumannii* Inhibits *C. albicans* Filamentation in *C. elegans*.** To determine the relative contribution of a bacterial secreted factor compared with live bacterial cells to the observed inhibition of *C. albicans* filamentation, we assessed the ability of *A. baumannii* culture filtrate, from different stages of growth, to inhibit *C. albicans* filamentation in *C. elegans*. We observed that filter-sterilized supernatant taken from *A. baumannii* grown to stationary phase inhibited *C. albicans* filamentation in *C. elegans*, however not to the level of live *A. baumannii* cells (Fig. 1B). Culture filtrate from exponential phase growth caused no inhibition (Fig. 1B). Supernatant from an environmental strain of *A. baumannii* strains OP50 or HB101, or the gram-positive pathogens *Enterococcus faecium* or *Staphylococcus aureus* (Fig. 1A).

The ability of *A. baumannii* to inhibit *C. albicans* filamentation was especially interesting. *A. baumannii* is an emerging, multidrug-resistant bacterial pathogen, and like *C. albicans*, has a predilection for infecting immunocompromised, critically ill patients (16). Both microbes often share a common ecological niche within healthcare institutions, including bronchial airways, vascular and urinary catheters, and patient wounds (14–17). Given the increasing importance of both of these opportunistic pathogens in the morbidity and mortality of hospitalized patients (8, 16), we sought to define further their interactions within *C. elegans*. First, we assessed a range of *Acinetobacter* species, and found that the nonpathogenic species, *Acinetobacter baeyli*, and the unusual human pathogen, *Acinetobacter lwoffi*, inhibited *C. albicans* filamentation significantly less well than *A. baumannii* [supporting information (SI) Fig. S1]. Interestingly, *Acinetobacter calcoaceticus*, a common environmental organism (16), inhibited *C. albicans* filamentation to a similar extent as *A. baumannii*, which may be as a consequence of its evolutionary development with environmental fungi in nature, leading to the formation of inherent mechanisms for competitive survival. Notably, five other clinical *A. baumannii* strains also inhibited *C. albicans* filamentation (data not shown).

The observed *A. baumannii*–*C. albicans* interaction within *C. elegans* remained robust despite modifying the environment of the coinfection assay with factors that promote *C. albicans* filamentation. The *A. baumannii*-mediated inhibition of filamentation was not altered by bovine serum (at different concentrations up to 75%) (Fig. 1A). Asterisks denote comparison with *C. albicans* strain DAY185 alone: ***, P ≤ 0.001; **, P ≤ 0.01; *, P ≤ 0.05, by two-tailed t test. (Scale bars: C, D, and G, 50 μm; F, 20.35 μm; H, 45.84 μm; I, 13.85 μm; J, 21.89 μm.)
A. baumannii was recently identified as having antifungal activity (20). Isomers of iturin A were identified as the active molecules. Thus, we hypothesized that either acyl-homoserine lactone (AHL) synthases (3-hydroxy-C12-homoserine lactone) or 3-oxo-C12-homoserine lactone (Caymen Chemical) up to 200 μM, in the C. elegans–C. albicans infection model. No inhibition of C. albicans filamentation in C. elegans was observed (data not shown).

**A. baumannii Attenuates C. albicans Pathogenicity in the C. elegans Coinfection Model.** Given the importance of filamentation in the pathogenesis of *C. albicans* infection in mammals (10, 13) and *C. elegans* (9), we sought to assess the consequences of the observed *A. baumannii–C. albicans* interaction on the pathogenicity of *C. albicans* toward *C. elegans*. We hypothesized that, despite *A. baumannii* (22) and *C. albicans* (9) being able to kill *C. elegans* individually, a combined infection might lead to attenuated killing compared with *C. albicans* alone. We observed that when *C. elegans* were sequentially infected for 2 h with *A. baumannii*, followed by *C. albicans* on separate BH agar plates, and then transferred into standard liquid medium, worm killing was significantly attenuated compared with that observed with *C. albicans* infection alone (*P* < 0.001) (Fig. 2). To remove uncertainty about whether the worm consumed *C. albicans* after *A. baumannii* exposure, we reversed the sequence of pathogen exposure. A similar attenuation in *C. elegans* killing was observed (*P* < 0.001) (data not shown).

**A. baumannii Affects the Viability of *C. albicans* In Vitro and Preferentially Associates with *C. albicans* Filaments.** To provide supportive evidence for the observed anticandidal effects of *A. baumannii* in the *C. elegans* model, and to further define the interaction, we performed *in vitro* coinfection cultures under planktonic conditions. Using the reference *C. albicans* strain DAY185 (Table S1), we found that *A. baumannii* caused significant killing of *C. albicans* over a 96-h period (Fig. 3A). Given that the *C. albicans* DAY185 strain can exist in both yeast and filamentous forms, and our interest in assessing whether *A. baumannii* toxicity toward *C. albicans* has morphological specificity, we assessed the viability, in the same coinfection assay, of the constitutively filamentous *C. albicans* tup1 mutant (23) and the hyphal-defective mutant *suv3* (Fig. 3B). The white arrow in *E* points to a *C. albicans* filament, whereas the black arrow points to a yeast cell. Cocultures with *A. baumannii* and *C. albicans* *suv3* (solid line) was significantly reduced compared with the respective controls. A. baumannii caused more rapid killing of the constitutively filamentous *C. albicans* tup1 mutant (dotted line) compared with the hyphal-defective *C. albicans* mutant *suv3* (gray solid line), which remains in yeast form at 30°C but can produce filaments at 37°C. In support, microscopy showed that *A. baumannii* cells have greater association with *C. albicans* filaments compared with the yeast form, *tup1* (C, *suv3* at 30°C (D), and *suv3* at 37°C (E). The white arrow in *E* points to a *C. albicans* filament, whereas the black arrow points to a yeast cell. Cocultures with *A. baumannii* and *C. albicans* *suv3* (F) and *tup1* (G) mutants were stained with the LIVE/DEAD staining system. Viable cells stained green (SYTO9), whereas dead cells stained red (propidium iodide). Control cultures of *C. albicans* *tup1* and *suv3* mutants alone demonstrated green fluorescence (data not shown). All images were taken at 16–20 h. Asterisks denote comparison of Log CFU/ml between *C. albicans* *tup1* and *suv3* mutants when cocultured with *A. baumannii*. Results for the viability assays were derived from three independent experiments. (Scale bars: C, 6.87 μm; D, 6.35 μm; E, 6 μm; F, 8.75 μm; G, 12.54 μm.)
The Complex Interplay Between Competing Pathogens: The Eukaryotic Quorum-Sensing Molecule, Farnesol, Inhibits the Growth of A. baumannii. To further explore the counteroffensive by C. albicans toward A. baumannii in a biofilm environment, we assessed the supernatant from the liquid medium of a C. albicans biofilm at different stages of development for its activity against A. baumannii. We observed that the antibacterial activity of the C. albicans supernatant increased as biofilm development matured. More specifically, significant A. baumannii growth inhibition (1.85 Log CFU/ml) was seen when grown in supernatant from an 18-h C. albicans biofilm. (P < 0.01) (Fig. S3A). Given the growth-dependent characteristics of these findings, we hypothesized that C. albicans quorum-sensing molecules may be responsible for the inhibitory activity against A. baumannii. Farnesol appeared an ideal candidate for this finding as it is produced in parallel to C. albicans cell growth, its activity increases during the later stages of biofilm development (26), and it has been described to have antibacterial activity (27). Indeed, we showed that pure farnesol (Sigma) caused a significant inhibition of A. baumannii growth (Fig. S3B), of similar magnitude to that seen with C. albicans supernatant. To further confirm these findings we assessed the supernatant from a mature biofilm of a C. albicans mutant defective in farnesol production (C. albicans KWN2) and compared its effect on A. baumannii growth to the supernatant of its parent strain (C. albicans SN152) and a reconstituted strain (C. albicans KWN4) (28). Supernatant from C. albicans KWN2 caused no inhibition of A. baumannii growth compared with A. baumannii grown in fresh medium, whereas C. albicans SN152 and KWN4 caused a subtle yet significant reduction in A. baumannii growth (0.59 and 0.51 Log CFU/ml reduction, respectively, P < 0.01 for both). These data confirm that the eukaryotic quorum-sensing molecule, farnesol, has crosskingdom inhibitory effects on the prokaryotic organism, A. baumannii.

Fig. 4. A. baumannii and C. albicans in an in vitro biofilm environment. (A) The degree of inhibition of C. albicans DAY185 (CA) biofilm formation on silicone pads depended on the initial A. baumannii (AB) inoculum. (B) A. baumannii culture filtrate, when used as the liquid medium for the assay, inhibited C. albicans biofilm formation, with the degree of inhibition depending on the bacterial growth phase (grown in spider media at 37°C) from which the supernatant (SUP) was derived (OD at 600 nm). When A. baumannii was introduced into the liquid medium of the biofilm assay after 8 h of C. albicans biofilm development (C), further biofilm growth was inhibited. However, when A. baumannii was inoculated after 24 h, the inhibitory effects were reduced, with evidence of further C. albicans biofilm growth (D). LIVE/DEAD staining of a mature (60 h) C. albicans biofilm in the presence of A. baumannii (D) showed viability only of yeast cells. (Scale bar: D, 17.49 μm.) Asterisks in A denote comparison of biofilm mass (mg) between C. albicans alone and in the presence of A. baumannii, and the asterisks in B denote comparison of biofilm mass between C. albicans alone in standard spider media compared with A. baumannii supernatant as the media of the assay. Experiments were performed at least twice in triplicate.

C. elegans killing (9). Given the importance of functional C. albicans filaments to biofilm integrity (12), we assessed the consequences of the A. baumannii–C. albicans interaction on C. albicans biofilm formation, using a silicone pad assay (12). When A. baumannii was introduced into the media of the biofilm assay, a significant dose-dependent inhibition of C. albicans biofilm formation was observed (Fig. 4A). Microscopy at the completion of the experiment showed that the sparse biofilm that formed was composed mainly of C. albicans cells that had yeast morphology (Fig. S2A and B). Interestingly, C. albicans filaments in the mature biofilm were nonviable in the presence of A. baumannii, as determined by the LIVE/DEAD staining system (Fig. 4D). Of note, culture filtrate from A. baumannii also significantly inhibited the ability of C. albicans to form a biofilm, with the degree of inhibition being dependent on the A. baumannii growth phase in which the culture filtrate was taken (Fig. 4B). To assist in ruling out the possibility that the reduction of C. albicans biofilm on silicone pads was because of the depletion of some nutrient from the medium, we evaluated the C. albicans biofilm in the presence of less pathogenic species of Acinetobacter, including A. baumannii and A. hofii. These strains were unable to inhibit C. albicans biofilm formation (data not shown).

To determine the effect of A. baumannii on an already formed C. albicans biofilm, we inoculated the biofilm environment with live A. baumannii cells at different stages of C. albicans biofilm development. We observed that when A. baumannii was inoculated at a cell density of 10⁶ CFU/ml up to 8 h after C. albicans biofilm development, the ability of further C. albicans biofilm formation was inhibited (Fig. 4C and data not shown). Unexpectedly, we noticed that there was a time point beyond which the effect of A. baumannii cells toward C. albicans decreased. More specifically, when A. baumannii was introduced into the C. albicans biofilm assay after 24 h of C. albicans biofilm formation, not only was it less able to inhibit further biofilm development (Fig. 4C) but interestingly, the growth of the bacteria appeared restricted.

A. baumannii Mutants with Attenuated Virulence toward C. albicans Identified by Using a C. elegans–A. baumannii–C. albicans Screen. To determine whether the C. elegans–A. baumannii–C. albicans coinfection model can be used to explore the molecular mechanisms of this prokaryote–eukaryote interaction, we extended our model to allow for analysis in 96-well plates of ~600 random A. baumannii MAR2xT7 transposon mutants. After performing confirmatory assays, five mutants were identified that had significantly less ability to inhibit C. albicans filamentation in C. elegans. All five mutants had similar growth kinetics to the parent strain (data not shown). The insertion site of one of these mutants was identifiable using gene sequence homology to the gacS-like sensor kinase gene, which is part of a highly conserved two-component regulatory system (GacS sensor kinase/GacA response regulator) important for a diverse array of virulence functions in other gram-negative bacteria (29, 30). Interestingly, for many gram-negative bacteria, the gacS/gacA two-component system has been shown to control the synthesis of secretory products, including secondary metabolites with antimicrobial activity (29). Further, a P. aeruginosa gacA mutant, which is attenuated in virulence toward mammals (30), was found to be delayed in its inhibitory effect toward C. albicans (4). Apart from causing significantly less inhibition of C. albicans filamentation in C. elegans, the A. baumannii gacS-like sensor kinase mutant was also attenuated in its ability to kill the C. albicans DAY185 strain in vitro (Fig. S4). With regard to the insertion sites of the other four mutants, one was identified within a gene coding for a hypothetical protein and the last three mutants were not identifiable.

Discussion

Given the abundance of polymicrobial encounters in nature, and the paucity of knowledge about the pathogenic consequences and
molecular details of these interactions, we developed a facile *in vivo* whole animal model that can be effectively used to study pathogen–pathogen interactions. Using this model system, we found that important virulence traits of *C. albicans*, such as biofilm and filament formation, are targets of *A. baumannii*. Interestingly, *A. baumannii* cells have a greater affinity and toxicity toward *C. albicans* filaments compared with the yeast form. Remarkably, although bacteria inhibit *C. albicans* biofilm formation, when allowed to develop, the growth of *A. baumannii* is inhibited. This likely evolutionary defense system by *C. albicans* is at least partly because of the release of the quorum-sensing molecule farnesol.

Moreover, although *A. baumannii* and *C. albicans* can independently kill *C. elegans*, when nematodes are infected with both pathogens they survive significantly longer compared with *C. albicans* infection alone. Finally, in a “proof of concept” study, we screened *A. baumannii* mutants and identified those with reduced toxicity toward *C. albicans*.

Thus far, there is a scarcity of realistic *in vivo* models that exist to study pathogen–pathogen interactions (6). The *C. elegans* model, which has thus far been used to study monomicrobial pathogenesis (7), provides many advantages for the study of multispecies dynamics, including genetic tractability, ease of handling and simplicity of equipment, short reproductive cycle, translucent body that enables microscopic visualization of internal events, and absence of ethical considerations associated with mammalian models. Also, for the study of the prokaryote–eukaryote interactions described herein, two relatively unambiguous assay endpoints are used: *C. albicans* filamentation and worm survival. In addition, the ability to genetically manipulate both the host and the pathogen provides an efficient system to study the molecular mechanisms of pathogen–pathogen interactions and host responses to polymicrobial infections. Such models will help advance our understanding of microbrial pathogenesis within a realistic environment of coexisting microbes.

Our results show that *A. baumannii* has profound antifungal properties, with a predilection for filamentous forms of *C. albicans*. Filamentation has been shown to be an important virulence determinant in *C. albicans* (10, 13), and thus our findings of reduced *C. albicans* pathogenicity toward *C. elegans* when coinfected with *A. baumannii*, are understandable. Recently, our group demonstrated that *C. elegans* killing was reduced when infected with *C. albicans* mutants defective in hyphae formation (9), thus highlighting the relevance of filamentation in the *C. elegans* infection model. However, the reduction in nematode killing by *C. albicans* in the presence of *A. baumannii* is likely because of a broader effect on *C. albicans*, including the toxicity of *A. baumannii* to the yeast-form cells as well. After *C. elegans* consumes *C. albicans*, a persistent gut infection ensues, initially composed of yeast-form cells. These cells proliferate and cause marked gut distension followed by a morphological transition to the filamentous form, eventually leading to worm death (9). Given the observed effects of *A. baumannii* on the viability of *C. albicans* yeast and filamentous forms, it is reasonable to assume that *A. baumannii* slows or reduces the degree of *C. albicans* proliferation in the worm gut, thus reducing worm lethality. Also, it is possible, however not assessed in this study, that an augmented or altered host immune response with polymicrobial infection may favor worm survival.

The cause of the antagonistic interaction between *A. baumannii* and *C. albicans* appears multifactorial. It is clear that a bacterial secretory factor, whose production increases from late exponential growth phase onwards, plays a significant role. However, inhibition of *C. albicans* filamentation and biofilm formation was more pronounced with live *A. baumannii* cells compared to supernatant taken from stationary phase growth, suggesting that cellular interaction may also contribute. Also, microscopy demonstrated marked cell–cell association of *A. baumannii* with the filamentous cells of *C. albicans*, further raising the question of direct cellular toxicity. Recently, Smith et al. performed whole genome sequencing of a reference strain of *A. baumannii* (American Type Culture Collect [ATCC] strain no. 17978) and identified eight genes homologous to the Legionella/Coxiella Type IV secretion apparatus (22). This secretion system is capable of exporting virulence factors across the membranes of gram-negative bacteria, often to eukaryotic cell targets (31). Thus, the type of secretion may be used by *A. baumannii* for direct toxicity toward *C. albicans*. Also, the close cellular association of *A. baumannii* to *C. albicans* that we observed likely optimizes the potency of a secreted factor with antifungal activity.

Other factors that may be contributing to the observed antagonistic interaction between *A. baumannii* and *C. albicans* include changes in environmental pH, which can impair the ability of *C. albicans* to form filaments (32) and nutritional competition. We observed that *A. baumannii* can produce a mildly alkalotic environment under the conditions of the *C. elegans* coinfection assay but that this was not sufficient to cause appreciable inhibition of *C. albicans* filamentation (data not shown). With regard to nutrient depletion, we observed that *C. albicans* filamentation in the presence of *A. baumannii* remained significantly inhibited despite frequent (6 hly) replenishment of the liquid media with fresh BHI medium during the *C. elegans* coinfection filamentation assay. Moreover, the inhibition of filamentation occurred rapidly, before one would expect the depletion of nutrients to occur, and *A. baumannii* showed a cellular predilection for *C. albicans* filaments, suggesting an interaction beyond just nutrient deprivation.

The culture filtrate from *A. baumannii* grown to stationary phase significantly inhibited *C. albicans* filamentation and biofilm formation. Given the growth-dependent properties of this antifungal activity, a quorum-sensing molecule may be a likely etiological candidate. However, we were unable to demonstrate this by using an *A. baumannii* mutant defective in the production of 3-hydroxy-C12 homoserine lactone (abel::Kan) (21) and a range of synthetic AHL molecules with varying carbon-length backbones. The production of another cell-density-dependent compound is also a possibility, as has been shown for an environmental strain of *Acinetobacter* toward phytopathogenic fungi, which produced iturin A (20). In our study, iturin A was not effective at inhibiting *C. albicans* filamentation in *C. elegans* up to a concentration of 5 μM. Given the therapeutic potential of our findings, further work to identify the active compound in *A. baumannii* supernatant is ongoing.

Remarkably, we observed a counteroffensive by *C. albicans* toward *A. baumannii* within the complex environment of a mature biofilm. We identified that a secretory factor with antibacterial activity was being released from *C. albicans* toward the later stages of biofilm development. Through use of purified farnesol and a *C. albicans* mutant defective in farnesol production (28), we confirmed that this eukaryotic quorum-sensing molecule, which represses hyphae formation, was at least partly responsible for the observed antibacterial effect against *A. baumannii*. Interestingly, farnesol is a molecule with a 12-carbon backbone chain length, similar to certain bacterial quorum-sensing molecules (32). Such crosskingdom targets of extracellular signaling illustrates the diversity of these molecules and highlights their potential in uncovering novel therapeutic targets for clinically problematic pathogens.

In conclusion, this report extends the current paradigm of studying monomicrobial pathogenesis in the genetically tractable, whole animal model system, *C. elegans*. The evolution of synergistic, symbiotic, or antagonistic interactions between diverse organisms in nature or the clinical environment, especially those between prokaryotes and eukaryotes, is likely important for their pathogenesis toward a range of hosts, including humans. The exploitation of the likely evolutionary defense mechanisms used by competing microbes may provide critical insights into novel therapeutic targets, which are desperately needed for pathogens such as *A. baumannii* and *C. albicans*. 

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Note: The reference list and corresponding citations are not included in the natural text representation above.
Experimental Procedures

Bacterial and Fungal Strains. Unless specified otherwise, bacterial and fungal cultures were grown overnight in Luria–Bertani (LB) broth at 37°C and yeast peptone dextrose (YPD) (Difco) broth at 30°C in a rollerdrum, respectively. The genotypes and other characteristics of C. albicans strains used in this study are reported in SI Text and Table S1. Heat-killed A. baumannii were produced by incubating cells in a heat block at 80°C for 90 min.

C. elegans Strains. C. elegans glp-4; sek-1 nematodes were used for all experiments because of the untoward effects of using wild-type C. elegans strains in liquid assay experiments (see SI Text). The glp-4; sek-1 nematodes were propagated on E. coli strains OP50 or HB101 by using established procedures (9).

C. elegans Coinfection Assay for Filamentation. The methodology used for the C. elegans–C. albicans liquid medium assay was as described previously (9), with some modification. Young adult nematodes were allowed to feed on lawns of C. albicans on solid BHI media (Difco), containing kanamycin (45 μg/ml), ampicillin (100 μg/ml), and streptomycin (100 μg/ml), for 4 h at 25°C (preinfection). The worms were then washed with sterile M9 minimal media and pipetted (approximately 60 to 80 worms per well) into wells of a six-well microtiter dish (Corning) containing 2 ml of liquid media (80% M9 and 20% BHI). Bacteria from overnight culture were directly inoculated into the liquid media immediately before the preinfected worms were included. Plates were incubated at 25°C and examined daily for the number of worms with filamentation. By using a Nikon SMZ645 dissecting microscope. Filamentation was defined as any breach in the worm cuticle by filamentous cells as seen at X50 magnification

Differences in worm filamentation on day 5 were compared by the Student’s t test. For this and all subsequent statistical comparisons, a P value of <0.05 was considered statistically significant. Qualitative assessment of C. albicans filamentation was performed by confocal laser microscopy (TCS NT; LeicaMicrosystems). All experiments were performed at least twice.

C. elegans Coinfection Assay for Survival. Nematodes were preinfected with both organisms sequentially for 2 h on solid medium (BHI) before being transferred into liquid medium (M9:BHI as described). To remove excess bacterial or fungal cells from the worm cuticle, nematodes were briefly washed with M9 and then into liquid medium (M9:BHI as described). To remove excess bacterial or fungal cells from the worm cuticle, nematodes were briefly washed with M9 minimal media and pipetted (approximately 60 to 80 worms per well) into wells of a six-well microtiter dish (Corning) containing 2 ml of liquid medium (80% M9 and 20% BHI). Bacteria from an overnight culture were directly inoculated into the liquid medium immediately before the preinfected worms were included. Plates were incubated at 25°C and examined daily for the number of worms with filamentation. By using a Nikon SMZ645 dissecting microscope. Filamentation was defined as any breach in the worm cuticle by filamentous cells as seen at X50 magnification.

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In Vitro Coinfection Cultures in a Planktonic Environment. Coinfection cultures were performed in 2 ml of LB broth at 30°C in a rollerdrum. YPD plates containing kanamycin (45 μg/ml) and LB plates containing fluconazole (32 μg/ml) were used to determine C. albicans and A. baumannii CFUs, respectively. Results were obtained from three independent experiments. The viability of C. albicans in the presence of A. baumannii was also assessed by using the BacLight LIVE/DEAD staining system according to the manufacturer’s protocol (Molecular Probes) (32).

In Vitro Biofilm Assay on Silicone Pads. The effect of A. baumannii on C. albicans biofilm formation was determined by using a silicone pad assay as described previously (11, 12) and detailed in SI Text. To assess the effects of Acinetobacter on C. albicans biofilm formation, bacterial cells were introduced into the biofilm medium at different time points. Silicone pads exposed to Acinetobacter only were used as a control. Results were obtained from at least two independent experiments performed in triplicate.

Development of the A. baumannii MAR2X7 Mutant Library. MAR2X7 inserts were generated by introducing pMAR2X7 into a gentamicin-susceptible A. baumannii clinical strain A9844 from E. coli/MC4100 in six separate triplicate matings as described previously (34), with slight modification (SI Text). Form species identification of A9844 was performed before mutagenesis (SI Text).

Screening Using the C. elegans–A. baumannii–C. albicans Model. An A. baumannii library consisting of approximately 600 MAR2X7 mutants was screened for their ability to inhibit C. albicans filamentation in C. elegans. First, A. baumannii mutants were replicated into 96-well microtiter plates containing fresh LB media with 15 μg/ml of gentamicin and allowed to grow overnight at 37°C. Nematodes were then preinfected with C. albicans and then pipetted into each well of the A. baumannii microtiter plates (approximately 30 to 40 worms per well). The percentage of worms with filamentation was assessed on day 5. Mutants that allowed ≥30% of the C. albicans preinfected worms to filament were tested two more times by using the standard assay. Transposon insertion sites were identified by using a nested PCR as described previously (34).

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3. Hansen SK, Rainey PB, Haagensen JA, Molin S (2007) Evolution of species interactions using a Nikon SMZ645 dissecting microscope. Filamentation was defined as any breach in the worm cuticle by filamentous cells as seen at X50 magnification

Differences in worm filamentation on day 5 were compared by the Student’s t test. For this and all subsequent statistical comparisons, a P value of <0.05 was considered statistically significant. Qualitative assessment of C. albicans filamentation was performed by confocal laser microscopy (TCS NT; LeicaMicrosystems). All experiments were performed at least twice.
Supporting Information

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SI Experimental Procedures

**Bacterial and Fungal Strains.** *C. albicans* strain MLR62 (Fig. 1 G and H) has a GFP reporter linked to the constitutively active *TEF1* promoter (*ura3Δ::λimm434/ura3Δ::λimm434 his1::his/G/HS1-TEF1-GFP:: his1::hisG arg4::hisG/URA3::ARG4::arg4::hisG) (13) and thus expresses GFP when in the yeast or filamentous forms. *C. albicans* strain HGFP3 (Fig. 1I and J) (strain CA14 but with plasmid pHWP1GFP3 integrated at the *ENO1* locus) that expresses green fluorescence only in true hyphae (14).

For assessment of *A. baumannii* supernatant in the *C. elegans* model, bacterial cells were grown in the same media used for the nematode assay (80% M9 minimal media and 20% BH) at 37°C in a rollerdrum. The cells were spun down at exponential phase (4-h incubation) or at stationary phase growth (20-h incubation), and the resulting supernatant was filter-sterilized by using a 0.22 μm filter (Millipore). Two milliliters of this culture filtrate were then used as the liquid medium for the *C. elegans* assay. The absence of bacterial cells was confirmed by plating the supernatant on LB agar.

**C. elegans Strains.** As described previously (1), progeny production by wild-type *C. elegans* strains can lead to untoward effects in *C. elegans*-*C. albicans* liquid assay experiments, including difficulties with scoring the viability of infected parents and significant matricidal death (premature hatching of eggs in the *C. elegans* uterus leading to death that is not necessarily due to an infectious process). To avoid these difficulties, we used *C. elegans* *glp-4*; *sek-1* mutants that have normal morphology and brood sizes at 15°C but are unable to produce eggs at 25°C (1, 2). In the *C. elegans* imm434/ura3 strain (pRK2013 but with plasmid pHWP1GFP3 integrated at the *ENO1* locus) that expresses green fluorescence only in true hyphae (14).

**In Vitro Biofilm Assay on Silicone Pads.** In brief, pretreated silicone pads were exposed to 2 ml of spider medium (18) containing fresh overnight *C. albicans* cultures diluted to an OD_{600} of 0.5 and incubated in 12-well plates (Corning) at 37°C (shaking at 120–150 rpm) for 90 min. The silicone pads were then washed in PBS, placed in fresh spider medium, and incubated as above for 60 h. Quantitative biofilm assessment was performed by subtracting the original weight of the silicone pad from its postincubation weight and adjusting for the weight of a control pad exposed to no cells.

**Development of the A. baumannii MAR2xT7 Mutant Library.** A9844, MC4100, and a helper *E. coli* strain (pRK13) were mixed in a 1:2:2 ratio. Mixed cells were then washed in 500 μl of 100 mM MgSO_{4}, followed by 250 μl of fresh LB broth. A mix of A9844 plus MC4100 or pRK2013 was used as a negative control for all mating experiments. Multiple 25-μl mating spots were then placed on LB agar plates and allowed to incubate at 37°C overnight. Three mating spots were then transferred into 36 ml 100 mM MgSO_{4} and vortexed gently. One hundred twenty-five microliters of mating suspension were spread on each rectangular LB agar plate (Nunc Omnitray) and placed on LB agar plates and allowed to incubate at 37°C for 24 h. Two hundred fifty microliters of mating suspension were spread on each rectangular LB agar plate (Nunc Omnitray), and plates were incubated at 37°C for 24 h. Two hundred fifty microliters of mating suspension were spread on each rectangular LB agar plate (Nunc Omnitray), and plates were incubated at 37°C for 24 h.

**Supporting Information**

Fig. S1. Inhibition of *C. albicans* filamentation in *C. elegans* is dependent on the *Acinetobacter* species. The most clinically important and pathogenic strain, *A. baumannii* (ATCC strain no. 19606), caused the greatest degree of inhibition of *C. albicans* strain DAY185 filamentation. Other species include *A. calcoaceticus* ATCC strain no. 14987, *A. lwoffii* ATCC strain no. 15309, and wild-type *A. baylyi* strain ADP1 (PS8004) (7). All strains were inoculated into the liquid medium of the assay at a density of 10⁶ cfu/ml. Asterisks denote comparison of percentage of filamentation with *C. albicans* strain DAY185 alone.
Fig. S2. Confocal laser microscopy of *C. albicans* biofilm was performed in the absence (A) and presence (B) of *A. baumannii* with a X40 oil emersion objective and stained with Con A–Alexa 488 to identify *C. albicans* cell membranes (1). (Scale bars: 29.93 μm.)
Fig. S3. Farnesol inhibits the growth of *A. baumannii*. (A) *A. baumannii* growth (37°C) was inhibited when grown in filter-sterilized supernatant (SUP) taken from a more mature (>18 h) *C. albicans* DAY185 (CA) biofilm. (B) Farnesol (dashed column) at concentrations as low as 50 μM significantly (asterisks) inhibited *A. baumannii* growth compared with a DMSO control (solid column). Asterisks for A denote comparison of *A. baumannii* growth in fresh spider media compared with growth in *C. albicans* biofilm supernatant.
Fig. S4. The insertional *A. baumannii* gacS-like sensor kinase mutant is attenuated in killing *C. albicans* in vitro. The mean difference in *C. albicans* log cfu/ml over time between coinfection with the *A. baumannii* gacS mutant compared with coinfection with the parent strain (A9844) is represented. A positive value signifies a greater *C. albicans* log cfu/ml with the *A. baumannii* gacS mutant compared with A9844. The zero point on the y axis signifies no difference between the strains. Error bars represent the standard deviation. All experiments were performed in 2 ml of LB medium incubated at 30°C in a rollerdrum and were performed in triplicate.
Table S1. *Candida albicans* strains used in this study

<table>
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<tr>
<th>Candida albicans strain</th>
<th>Genotype</th>
<th>Source or reference</th>
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<tr>
<td>DAY185</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 his1::hisG/HIS1::his1::hisG arg4::hisG/URA3::ARG4::arg4::hisG</td>
<td>(8)</td>
</tr>
<tr>
<td>tup1 mutant</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 tup1::hisG/tup1::hisG-URA3-hisG arg4::hisG/arg4::hisG</td>
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<td>suv3 mutant</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 suv3::Tn7-URA3/suv3::Tn7-UAU1 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</td>
<td>(11)</td>
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<tr>
<td>nrg1 mutant</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 nrg1::hisG/nrg1::hph-URA3-hph arg4::hisG/arg4::hisG</td>
<td>Fink laboratory</td>
</tr>
<tr>
<td>efg1 mutant</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 efg1::hisG/efg1::hisG-URA3-hisG arg4::hisG/arg4::hisG</td>
<td>(12)</td>
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<tr>
<td>tec1 mutant</td>
<td>ura3Δ::λimm434/ura3Δ::λimm434 tec1::Tn7-URA3/tec1::Tn7-UAU1 his1::hisG/his1::hisG arg4::hisG/arg4::hisG</td>
<td>(13)</td>
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