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 crosskingdom 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 bacteria *A. baumannii* or *Pseudomonas aeruginosa*, filamentation by *C. albicans* was significantly inhibited (Fig. 1*A*). In contrast, filamentation was minimally affected by the nonpathogenic *Esche-*

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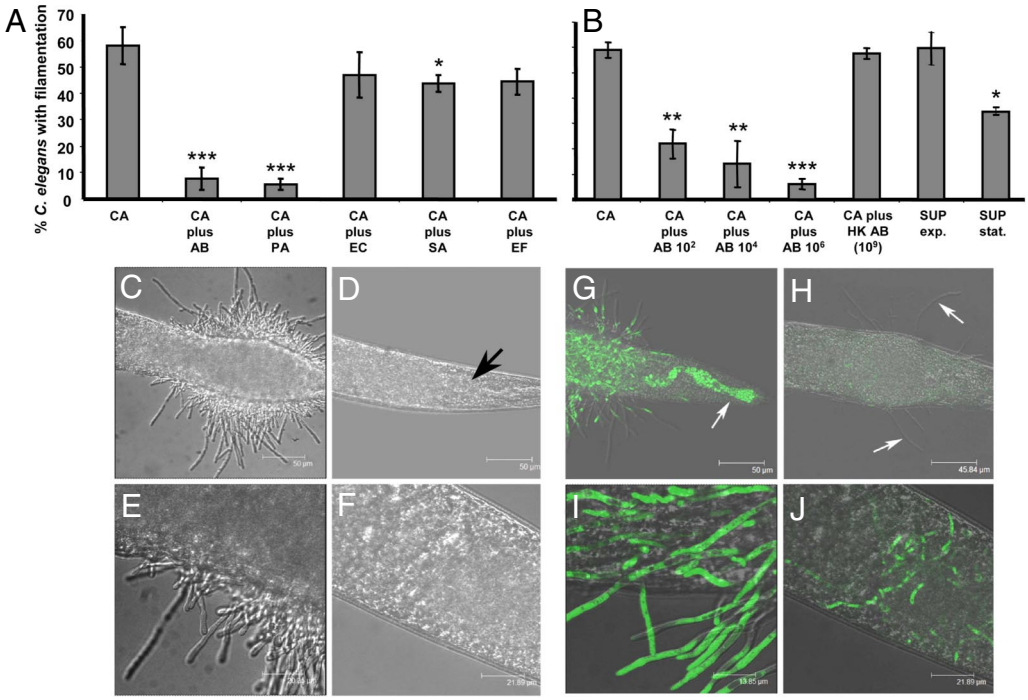

Fig. 1. Prokaryote–eukaryote interactions within *C. elegans*. (*A*) Inhibition of the reference *C. albicans* strain DAY185 (CA) filamentation depended on the coinfecting bacterial genus: *A. baumannii* (ATCC strain no.19606) (AB), *P. aeruginosa* strain PA14 (PA), *E. coli* strain OP50 (EC), *S. aureus* (ATCC strain no. 29213) (SA) and *E. faecium* strain A6349 (EF). All bacteria inocula were 10^6 cfu/ml. (*B*) Live *A. baumannii* cells, in a dose-dependent manner, and filter-sterilized supernatant (SUP) from stationary phase growth (stat.) were able to inhibit *C. albicans* filamentation. Heat-killed (HK) cells had no effect. Light-field microscopy images show the inhibition of filamentation in the presence of *A. baumannii* (*D* and *F*) compared with *C. albicans* alone (*C* and *E*) (black arrow in *D* points to the nematode grinder organ). Fluorescent images of *C. elegans* after exposure to *C. albicans* strain MLR62, which constitutively expresses GFP (*G* and *H*), shows yeast cells within the mouth and pharynx of the nematode (white arrow in *G*); with filamentation protruding from the distended proximal gut (*G*). In contrast, in the presence of *A. baumannii*, reduced GFP signal was observed, and if *C. albicans* filaments were present, they were attenuated (*H*, white arrows point to sparse filaments). Fluorescent images of *C. elegans* infected with the *C. albicans* strain HGFP3, which expresses GFP in a hyphae-specific manner (*I* and *J*), shows that *A. baumannii* inhibits true *C. albicans* hyphae (*J*), with few hyphae observed within the protective confines of the worm cuticle (*J*). All images were taken at 24 h. Column bars represent the mean and error bars represent standard deviation (for all figures). Asterisks denote comparison with *C. albicans* strain DAY185 alone: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$, by two-tailed *t* test. (Scale bars: *C*, *D*, and *G*, 50 μm; *E*, 20.35 μm; *F*, 21.89 μm; *H*, 45.84 μm; *I*, 13.85 μm; *J*, 21.89 μm.)

richia coli strains OP50 or HB101, or the gram-positive pathogens *Enterococcus faecium* or *Staphylococcus aureus* (Fig. 1*A*).

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 baylyi*, and the unusual human pathogen, *Acinetobacter lwoffii*, 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%) (Sigma), filament-inducing spider medium (18), the quorum-sensing molecule tyrosol (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. 1*B*). Washed, heat-killed *A. baumannii* cells at various densities (equivalent to 10^4–10^9 CFU/ml) caused no inhibition of *C. albicans* filamentation (Fig. 1*B* 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. 1*B*). Culture filtrate from exponential phase growth caused no inhibition (Fig. 1*B*). Supernatant from an environmental strain of *A. bau-*

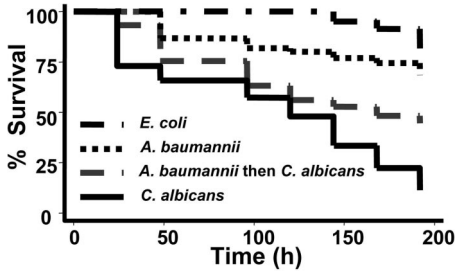

Fig. 2. *A. baumannii* attenuates the pathogenicity of *C. albicans* toward, *C. elegans*. The killing of *C. elegans* was significantly reduced when exposed to *A. baumannii* (ATCC strain no. 19606) and then *C. albicans* strain DAY185 (or vice versa) on solid medium before transfer to liquid medium, compared with exposure to *C. albicans* alone ($P < 0.001$). Nematodes feeding on *E. coli* OP50 were used as a control. Sixty to eighty nematodes were used per condition.

mannii was recently identified as having antifungal activity (20). Isomers of iturin A were identified as the active molecules. Thus, we tested pure iturin A (Sigma) in the *C. elegans–C. albicans* filamentation model and found that at concentrations up to 5 μM, no inhibition of filamentation was observed (data not shown), suggesting that antifungal entities are not limited to iturin A.

Given the growth-dependent activity of *A. baumannii* supernatant toward *C. albicans*, a further hypothesis was that a bacterial quorum-sensing molecule was responsible. Recently, the first of such molecules, a member of the LuxI family of autoinducer synthases (3-hydroxy-C_{12} homoserine lactone), was characterized from *A. baumannii* (21). After testing an *A. baumannii* mutant (*abaI*::K_m) defective in production of this molecule and its isogenic parent strain (M2) in the *C. elegans* coinfection model, we observed a similar degree of inhibition of *C. albicans* filamentation (data not shown), suggesting that either acyl-homoserine lactone (AHL) molecules are not responsible for our observations or that a different AHL molecule is responsible. To assess the latter further, we tested a range of synthetic AHL molecules with varying carbon backbone lengths, including C_4-, C_6-, and 3-oxo-C_{12}-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) (Fig. 2) 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 BHI 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*

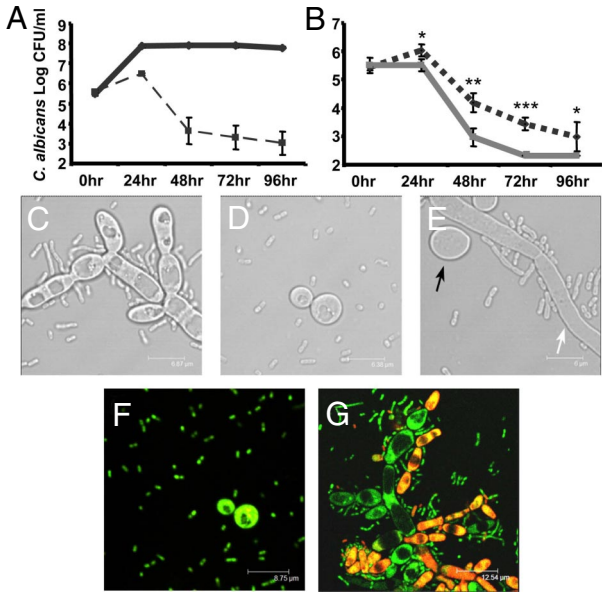

Fig. 3. *A. baumannii* has a predilection for killing *C. albicans* filaments *in vitro*. (*A*) The viability of *C. albicans* strain DAY185 (solid line) was significantly reduced when cocultured with *A. baumannii* (ATCC strain no. 19606) (dashed line). (*B*) *A. baumannii* caused more rapid killing 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.)

over a 96-h period (Fig. 3*A*). 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*, which remains in yeast form at 30°C (12). The *C. albicans tup1* mutant was remarkably susceptible to *A. baumannii* (Fig. 3*B*). In comparison, the *C. albicans suv3* mutant was also susceptible to *A. baumannii* but significantly less so than *C. albicans tup1* (Fig. 3*B*), indicating a predilection of *A. baumannii* for killing *C. albicans* filaments. In addition, microscopy demonstrated that *A. baumannii* have greater cell–cell association with *C. albicans* filaments compared with the yeast form (Fig. 3 *C–E*). Further, when coinfection cultures with *A. baumannii* and the *C. albicans tup1* and *suv3* mutants were assessed by using the LIVE/DEAD staining system, whereby live cells stain green (SYTO9) and dead cells stain red (propidium iodide), killing of *C. albicans* filaments by *A. baumannii* was observed, whereas this was not evident for yeast-form cells at the same time point (Fig. 3 *F* and *G*). This morphological predilection of *A. baumannii* for the filamentous form of *C. albicans* was further confirmed by other *C. albicans* mutants, whereby the constitutively filamentous *C. albicans nrg1* mutant (24) was killed significantly more than the *C. albicans efg1* or *tec1* mutants (11, 25) (Table S1), which are both in the yeast form at 30°C ($P < 0.01$ at 96 h time point) (data not shown).

***A. baumannii* Inhibits *C. albicans* Biofilm Formation on an Abiotic Surface.** *C. albicans* biofilm communities are thought to be critical in its pathogenesis toward humans (11, 12) and are also involved in

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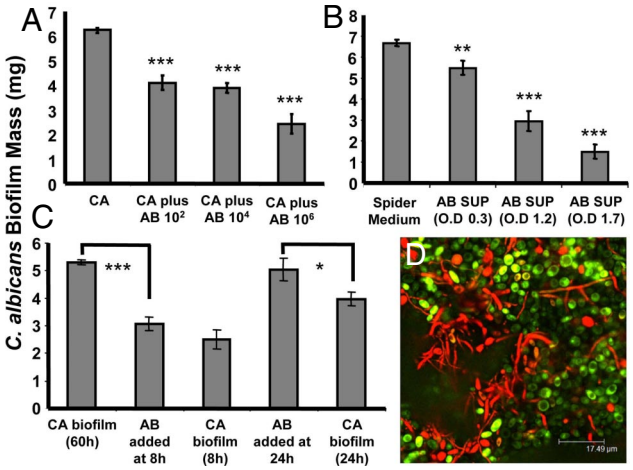

Fig. 4. *A. baumannii* and *C. albicans* in an *in vitro* biofilm environment. (*A*) The degree of inhibition of *C. albicans* DAY185 (CA) biofilm formation on silicone pads depended on the initial *A. baumannii* (AB) inoculum. (*B*) *A. baumannii* culture filtrate, when used as the liquid medium for 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 (*C*). 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 in *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. 4*A*). 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. S2 *A* 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. 4*D*). 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. 4*B*). 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. baylyi* and *A. lwoffii*. 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 10^6 CFU/ml up to 8 h after *C. albicans* biofilm development, the ability of further *C. albicans* biofilm formation was inhibited (Fig. 4*C* 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. 4*C*) but interestingly, the growth of the bacteria appeared restricted.

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. S3*A*). 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. S3*B*), 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*.

***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 microbial pathogenesis within a realistic environment of coexisting microbes.

Our results show that *A. baumannii* has profound anticandidal properties, with a predilection for the filamentous form 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 Collection [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, this type of system 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-C_{12} homoserine lactone (*abaI*::K_m) (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*.

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 an overnight culture were directly inoculated into the liquid media immediately before the preinfected worms were included. Plates were incubated at 25°C and were examined daily for the number of worms with penetrative 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 media between exposures to each organism. Worm death was monitored daily, and time to death was calculated by using the Kaplan–Meier method, with differences calculated by using the log-rank test (STATA 6).

***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 MAR2xT7* Mutant Library. *MAR2xT7* insertions were generated by introducing pMAR2xT7 into a gentamicin-susceptible *A. baumannii* clinical strain A9844 from *E. coli* MC4100 in six separate tripartite matings as described previously (34), with slight modification (*SI Text*). Formal 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 *MAR2xT7* 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 were then pipetted into each well of the *A. baumannii* preinoculated 96-well microtiter plates (approximately 30 to 40 worms per well). The percentage of worms with filamentation was assessed on day 5. Mutants that allowed $\geq$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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