EUKARYOTIC CELL, May 2009, p. 732–737 Vol. 8, No. 5
1535-9778/09/$08.00+0 doi:10.1128/EC.00016-09
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Interaction of *Candida albicans* with an Intestinal Pathogen, *Salmonella enterica* Serovar Typhimurium▽

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Received 11 January 2009/Accepted 18 March 2009

***Candida albicans* is an opportunistic human fungal pathogen that normally resides in the gastrointestinal tract and on the skin as a commensal but can cause life-threatening invasive disease. *Salmonella enterica* serovar Typhimurium is a gram-negative bacterial pathogen that causes a significant amount of gastrointestinal infection in humans. Both of these organisms are also pathogenic to the nematode *Caenorhabditis elegans*, causing a persistent gut infection leading to worm death. In the present study, we used a previously developed *C. elegans* polymicrobial infection model to assess the interactions between *S.* Typhimurium and *C. albicans*. We observed that when *C. elegans* is infected with *C. albicans* and serovar Typhimurium, *C. albicans* filamentation is inhibited. The inhibition of *C. albicans* filamentation by *S.* Typhimurium in *C. elegans* appeared to be mediated by a secretary molecule, since filter-sterilized bacterial supernatant was able to inhibit *C. albicans* filamentation. In vitro coculture assays under planktonic conditions showed that *S.* Typhimurium reduces the viability of *C. albicans*, with greater effects seen at 37°C than at 30°C. Interestingly, *S.* Typhimurium reduces the viability of both yeast and filamentous forms of *C. albicans*, but the killing appeared more rapid for the filamentous cells. The antagonistic interaction was also observed in a *C. albicans* biofilm environment. This study describes the interaction between two diverse human pathogens that reside within the gastrointestinal tract and shows that the prokaryote, *S.* Typhimurium, reduces the viability of the eukaryote, *C. albicans*. Identifying the molecular mechanisms of this interaction may provide important insights into microbial pathogenesis.**

Candida albicans, the most common human fungal pathogen, is a prototypical opportunistic organism that lives harmlessly in the human gastrointestinal tract but has the ability to cause life-threatening invasive disease. Bloodstream infection with *C. albicans* remains the most lethal form (10), with translocation of the gastrointestinal mucosa being an important pathogenic mechanism, especially in hemato-oncology patients and those who have undergone abdominal surgery. A key virulence determinant of *C. albicans* is its ability to transition from yeast to a filamentous form (16, 17, 19, 22). This morphogenesis appears important for tissue adherence and invasion (22). Furthermore, *C. albicans* has the ability to form complex biofilms on medical devices (13) and on human mucosal surfaces, such as the gastrointestinal and bronchial mucosa. *C. albicans* biofilm formation has immense clinical and economic consequences (13).

Recently the interactions between this important fungal pathogen and bacteria were described (11, 12, 18). These studies focus on the interaction between *C. albicans* and nonfermenting, gram-negative bacteria, such as *Pseudomonas aeruginosa* and *Acinetobacter baumannii*, whose interactions are likely found in the clinical environment, especially in the respiratory tracts of critically ill patients and on wounds of patients with burn injuries (7, 20). Of interest, these bacteria show antagonistic properties toward *C. albicans*, with a predilection toward reducing the viability of *C. albicans* filaments. In order to study these prokaryote-eukaryote interactions, our laboratory developed a polymicrobial infection model system using *Caenorhabditis elegans* as a substitute host (18). Previously, we showed that *C. albicans* causes a persistent lethal infection of the *C. elegans* intestinal tract (6). This leads to overwhelming *C. albicans* intestinal proliferation with subsequent filamentation through the worm cuticle (6). Given these characteristics, we decided to use this model to study the interaction of *C. albicans* with another intestinal pathogen, *Salmonella enterica* serovar Typhimurium.

S. Typhimurium is a gram-negative organism that belongs to the *Enterobacteriaceae* family. It is a gastrointestinal tract pathogen of humans, being responsible for approximately 2 million to 4 million cases of enterocolitis each year in the United States (4, 8, 21, 23). During infection, *S.* Typhimurium competes with normal intestinal flora (23). Its virulence pathways are well described, and it has been shown to cause a persistent and lethal gut infection of the nematode *C. elegans*, similar to infection seen with *C. albicans* (1, 14). Given this and the fact that *C. albicans* is a common inhabitant of the human gastrointestinal tract, we used the *C. elegans* polymicrobial infection model (18) to study the interactions between *S.* Typhimurium and *C. albicans*. Understanding the interactions between these diverse organisms within the complex milieu of an intestinal tract may provide important pathogenic and therapeutic insights.

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▽ Published ahead of print on 27 March 2009.

MATERIALS AND METHODS

Strains and media. Unless otherwise specified, fungal and bacterial cultures were grown overnight in yeast-peptone-dextrose (YPD) (Difco) broth at 30°C and LB broth at 37°C, respectively, and all experiments with *S.* Typhimurium were with the wild-type SL1344 strain, which is resistant to streptomycin and rifampin (rifampicin) (1). In the *C. elegans* assays, we used the green fluorescent protein-linked *S.* Typhimurium strain SL1344-GFP to help visualize the bacteria with confocal microscopy. This strain was resistant to ampicillin. Bacterial supernatant was filter sterilized using a 0.22-μm filter (Millipore) and was checked for sterility by plating on LB agar. *S.* Typhimurium supernatant was heat inactivated at 100°C for 30 to 45 min.

***C. elegans* coinfection assay for Filamentation.** The methodology for this assay was as described previously (18). In brief, *C. elegans glp-4;sek-1* nematodes were propagated on *E. coli* strain OP50 using established procedures. Synchronized, young adult nematodes were preinfected for 4 h on lawns of *C. albicans* and were then transferred, after washing in M9 medium, into wells of a six-well microtiter dish (Corning) (~70 to 80 nematodes per well). Each well contained 2 ml of standard liquid medium containing 80% M9 medium and 20% brain heart infusion. Just prior to transferral of the *C. albicans*-preinfected worms, *S.* Typhimurium was inoculated into the liquid media at various cell densities. The plates were then incubated at 25°C and examined at 24-h intervals for 5 days for the formation of penetrative filamentation using a Nikon SMZ645 dissecting microscope (TCS NT; Leica Microsystems). Penetrative filamentation was defined as any breach in the worm cuticle by filamentous cells as seen at magnification ×50. All experiments were repeated at least three times. Differences in worm filamentation on day 5 were compared by the Student *t* test. A *P* value of less than 0.05 was considered to be statistically significant. Qualitative assessment of *C. albicans* filamentation in *C. elegans* was performed using confocal laser microscopy (TCS NT; Leica Microsystems).

In vitro coinfection assay under planktonic conditions. In vitro coculture assays were performed in 2 ml of LB broth and were incubated in a roller drum at 30°C or 37°C, as appropriate. A starting inoculum of 10^6 CFU/ml of *S.* Typhimurium strain SL1344 and 5×10^5 CFU/ml of *C. albicans* was used for all experiments. YPD agar plates containing kanamycin (45 μg/ml), ampicillin (100 μg/ml), and streptomycin (100 μg/ml) and LB agar plates containing fluconazole (32 μg/ml) were used to select for *C. albicans* strains and *S.* Typhimurium, respectively. YPD and LB agar plates were incubated for 48 h at 30°C and 24 h at 37°C, respectively, before colonies were counted. Results were obtained from three independent experiments.

The viability of *C. albicans* when cocultured with *S.* Typhimurium was further assessed by using the Live/Dead staining system (Molecular Probes) according to the manufacturer's protocol.

Silicone pad biofilm assay. The effect of *S.* Typhimurium on *C. albicans* biofilm growth was evaluated using a polymicrobial silicone pad assay as described previously (18). Spider medium (10 g of nutrient broth, 10 g of mannitol, and 2 g of K_2HPO_4 in 1 liter) (15) was used as the medium for *C. albicans* biofilm development. The quantitative biofilm mass was calculated by subtracting the original weight of the silicone pad from its postincubation (60-h) weight and adjusting for the weight of a control pad exposed to no cells. All experiments were performed at least twice in triplicate. Differences in weight were determined by Student's *t* test, and a *P* value of <0.05 was considered to be statistically significant. Confocal laser microscopy was performed on mature biofilms.

RESULTS AND DISCUSSION

***S.* Typhimurium inhibits *C. albicans* filamentation in *C. elegans*.** Previously, our laboratory showed that pathogenic gram-negative organisms, such as *A. baumannii* and *P. aeruginosa*, are able to inhibit *C. albicans* filamentation in the *C. elegans co*infection model (18). This is in contrast to nonpathogenic gram-negative organisms, such as the auxotrophic *Escherichia coli* strain OP50, and gram-positive organisms, such as *Enterococcus* spp., which do not substantially inhibit *C. albicans* filamentation in *C. elegans* (18). After infecting young adult nematodes with *C. albicans* for 4 h and then transferring them into liquid medium containing 10^6 CFU/ml of *S.* Typhimurium, we observed a significant reduction in the proportion of worms with *C. albicans* filaments penetrating through their cuticles (Fig. 1). The effects were inoculum dependent, whereby larger numbers of bacterial cells caused greater inhibition of *C. albicans* filamentation (Fig. 1A). The antagonistic effect of *S.* Typhimurium on *C. albicans* is also shown by confocal microscopy, whereby a marked reduction in *C. albicans* filamentation was observed with coinfection with *S.* Typhimurium (Fig. 1D) compared to results without (Fig. 1C). Interestingly, at higher magnification, bacterial cells were seen adhering to the *C. albicans* filaments (Fig. 1E).

It has previously been shown that a secretory molecule from *P. aeruginosa* inhibits filament formation in *C. albicans* (12). To assess whether a bacterial secretory factor was responsible for the observed inhibition of *C. albicans* filamentation in *C. elegans* by *S.* Typhimurium, we transferred worms that had been infected with *C. albicans* into *S.* Typhimurium culture filtrate. As shown in Fig. 1B, when preinfected worms were transferred into bacterial supernatant taken from exponential-phase growth, no inhibition of *C. albicans* filaments was observed. However, when supernatant was taken from the stationary phase, significant inhibition of penetrative filamentation was seen (Fig. 1B).

These data suggest that *S.* Typhimurium has an antagonistic interaction with *C. albicans* and that a bacterial secretory factor plays a significant role in this process. Given the growth-dependent antifungal activity of *S.* Typhimurium culture filtrate on *C. albicans*, one may hypothesize that a quorum-sensing molecule may be responsible. However, secretion of a quorum-sensing molecule by *S.* Typhimurium has not been described thus far (2). Despite this, *S.* Typhimurium has been shown to have a quorum sensor encoded by *sdiA*, which responds to quorum-sensing molecules produced by other bacteria and can influence *S.* Typhimurium virulence (2, 3). We tested the effect of the *S.* Typhimurium *sdiA* mutant and found that this mutant caused the same degree of inhibition of *C. albicans* filamentation in *C. elegans* as its parent strain, 14028 (data not shown). Thus, the observed antifungal activity of *S.* Typhimurium supernatant is likely due to a different type of growth-dependent secretory molecule.

***S.* Typhimurium reduces the viability of *C. albicans* in vitro.** To further define the observed interaction between *C. albicans* and *S.* Typhimurium, we performed in vitro coinfection cultures under planktonic conditions. First, we coinfected the *C. albicans* reference strain DAY185 with the wild-type *S.* Typhimurium strain SL1344 in Luria-Bertani (LB) medium at temperatures closer to that used in the *C. elegans* model (30°C). As shown in Fig. 2A, after initial growth of *C. albicans*, the viability of the fungus declined in the presence of *S.* Typhimurium. Interestingly, when the coinfection culture was incubated at temperatures more consistent with those of the human body (37°C), the killing of *C. albicans* by *S.* Typhimurium was significantly more rapid, with undetectable levels by 48 h (Fig. 2A). Of note, the growth of *C. albicans* and that of *S.* Typhimurium when cultured alone were similar when incubated at 30°C and 37°C (data not shown).

These data suggest that either *S.* Typhimurium is more pathogenic or *C. albicans* is more susceptible at 37°C. Temperature is an important environmental stimulus for *C. albicans* morphogenesis: at 37°C the majority of *C. albicans* cells form filaments while at 30°C there is only a small ratio of hyphae to yeast cells (9). Given our observations of *C. albicans* filament inhibition in coinfection with *S.* Typhimurium in *C.*

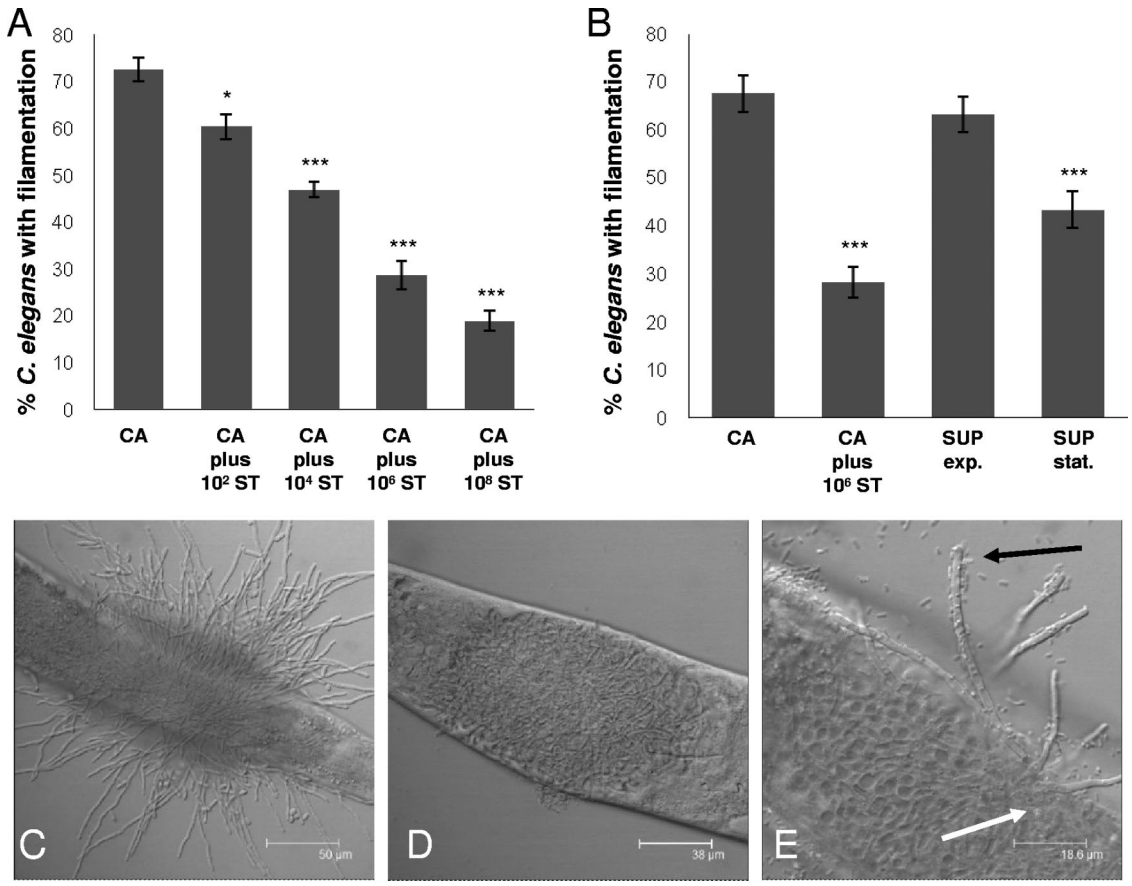

FIG. 1. *S.* Typhimurium (ST) inhibits *C. albicans* (CA) filamentation in the *C. elegans co*infection model. (A) The degree of inhibition of *C. albicans* filamentation was dependent on the initial inoculum of *S.* Typhimurium in the liquid medium of the assay. (B) *S.* Typhimurium supernatant taken from stationary-phase growth (16 h of growth) (SUP stat.) caused a significant inhibition of *C. albicans* filamentation in the *C. elegans* model, whereas that taken from exponential-phase growth (4 h of growth) (SUP exp.) did not. All images were taken at 24 h. Column bars represent the means, and error bars represent the standard deviations. Confocal microscopy showed the marked phenotypic differences between *C. elegans* infection with *C. albicans* alone (C) and infection with *C. albicans* and *S.* Typhimurium (D). (E) By using a green fluorescent protein-linked *S.* Typhimurium strain (SL1344-GFP), we observed not only bacteria and fungal cells within the gut of the worm (white arrow) but also bacteria associating with sparse filaments that had protruded through the worm cuticle (black arrow). Asterisks denote comparison of percentage filamentation with that for *C. albicans* strain DAY185 alone: ***, $P \leq 0.001$; **, $P \leq 0.01$; *, $P \leq 0.05$ (two-tailed *t* test). Scale bar: 50 μm (C), 38 μm (D), or 18.6 μm (E).

elegans and the temperature differences in the viability of *C. albicans* in the coinfection in vitro cultures, we tested whether *S.* Typhimurium has morphological specificity in its antagonism toward *C. albicans*. Furthermore, we have previously shown that *A. baumannii* has a predilection for killing the filamentous form of *C. albicans* (18). Using the in vitro assay already described, we coinfected the constitutively filamentous *C. albicans tup1* mutant (5, 11) and the *C. albicans suv3* mutant, which is impaired in hypha formation and remains in the yeast form at 30°C (18, 19), with *S.* Typhimurium (SL1344) at 30°C. As shown in Fig. 2B, killing was observed for both *C. albicans* mutant strains; however, it was more pronounced for the *C. albicans tup1* mutant by 96 h of incubation. More-rapid killing was observed with both mutant strains when the experiment was repeated at 37°C (data not shown). Given that the *suv3* mutant can form filaments at 37°C, we were unable to detect a statistically significant difference between the killing of these strains. Taken in their totality, these data suggest that the temperature effect may be associated with *C. albicans* morphology or it could be independent of morphology and associated with increased susceptibility of *C. albicans* at 37°C. Nevertheless, increased pathogenicity of *S.* Typhimurium against *C. albicans* at 37°C cannot be excluded.

Interestingly, when we performed confocal microscopy of the in vitro coinfection culture with the reference strain of *C. albicans* (DAY185) (Fig. 3C) and the *C. albicans tup1* mutant (Fig. 3G), we did not see significant cell-cell association as has been shown previously with *A. baumannii* and *P. aeruginosa* (11, 18). However, after the coculture was stained at 24 h (incubation at 37°C) with the Live/Dead staining system (Molecular Probes), whereby live cells stain green (SYTO9) and dead cells stain red (propidium iodide), the viability of the filaments was reduced from that of the yeast form (Fig. 3D and H). These data suggest that a secretory molecule may be the predominant mechanism by which *S.* Typhimurium kills *C. albicans*.

To determine the timing in the bacterial growth phase in which the *S.* Typhimurium supernatant reduced the viability of

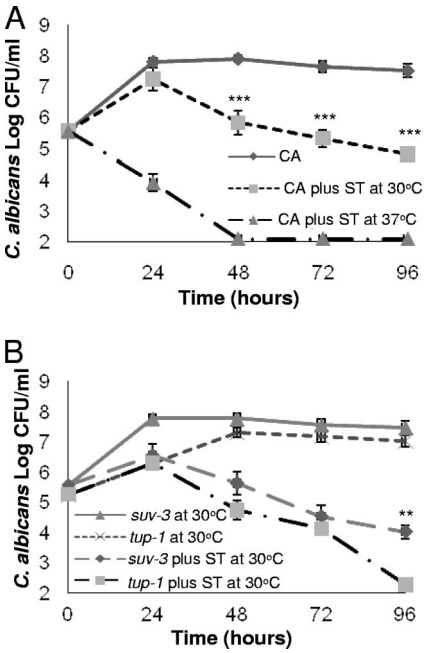

FIG. 2. *S.* Typhimurium (ST) reduces the viability of *C. albicans* (CA) in an in vitro environment. (A) The viability of the *C. albicans* DAY185 strain in the presence of *S.* Typhimurium is significantly lower at 37°C than at 30°C. The *C. albicans suv3* mutant, which is in the yeast form at 30°C, was more resistant to killing by *S.* Typhimurium than the constitutively filamentous *C. albicans tup1* mutant strain at 30°C (B). Error bars represent the standard deviations. In panel A, asterisks denote comparison of log CFU/ml of *C. albicans* DAY185 at 30°C and 37°C. In panel B, asterisks denote comparison of log CFU/ml of the *C. albicans suv3* strain to the *C. albicans tup1* strain at 30°C: ***, $P \leq 0.001$; **, $P \leq 0.01$ (two-tailed *t* test).

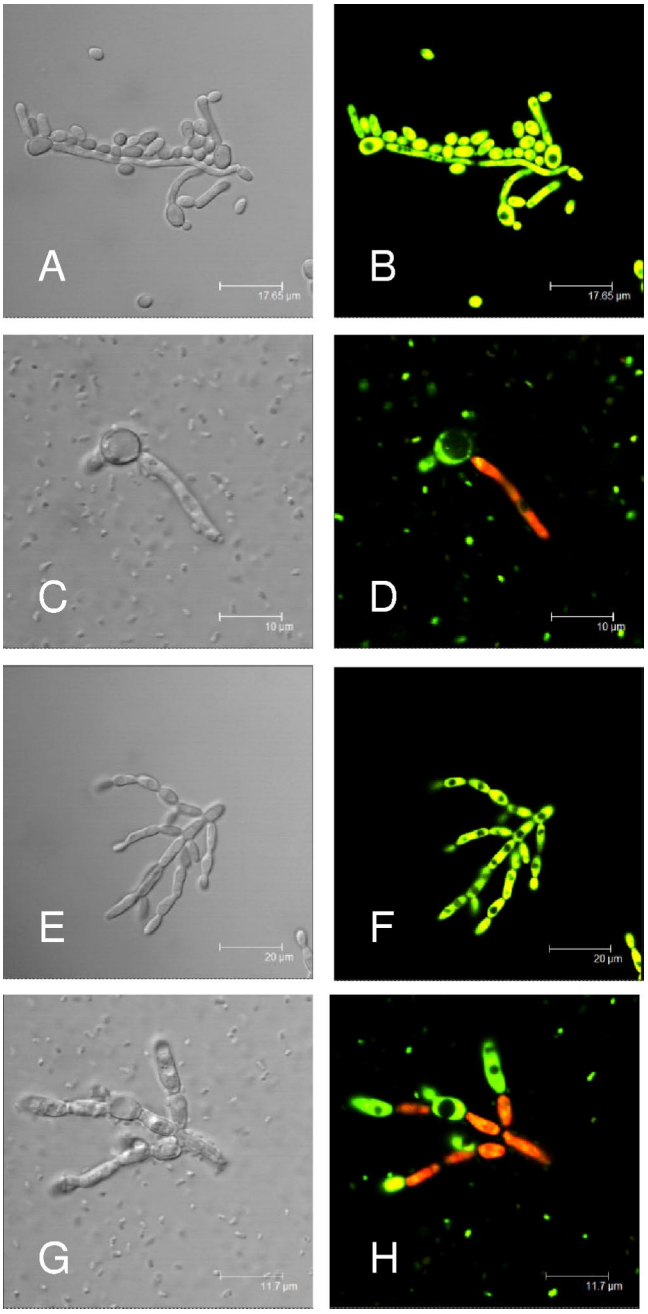

FIG. 3. Confocal laser microscopy of in vitro cultures after staining with the Live/Dead staining system, whereby dead cells stain red and live cells stain green. *C. albicans* strain DAY185 cultured alone (A and B) or in the presence of *S.* Typhimurium (C and D), showing a nonviable filamentous cell when cocultured with *S.* Typhimurium (D) despite an absence of pronounced cell-cell association. (E and F) The constitutively filamentous *C. albicans tup1* mutant cultured alone (E and F) or in the presence of *S.* Typhimurium (G and H), showing a similar finding of reduced viability (red stain) when cocultured with *S.* Typhimurium (H). Images were taken after 24 h of incubation in LB broth at 37°C. Scale bar: 17.65 μm (A and B), 10 μm (C and D), 20 μm (E and F), or 11.7 μm (G and H).

C. albicans, we cultured the fungus at 37°C in filter-sterilized supernatant taken from different stages of bacterial growth. As shown in Fig. 4A, the viability of *C. albicans* strain DAY185 was reduced when it was cultured in supernatant taken from at least 16 h of *S.* Typhimurium growth (stationary phase). The effects increased as supernatant was taken from bacteria grown for up to 24 h (Fig. 4A). In order to investigate further the effect of the supernatant on *C. albicans* filamentation, we inoculated the *C. albicans tup1* and *suv3* mutants in *S. Typhimurium* supernatant isolated from different time points of *S.* Typhimurium growth (in LB broth) and incubated them at 37°C. Interestingly, the constitutively filamenting *tup1* mutant was more susceptible to the bacterial supernatant isolated at 12 h (early stationary phase) than the *C. albicans suv3* mutant (Fig. 4B and C). Both strains were inhibited in supernatants isolated from late stationary phase. Given the fact that an acidic environment decreases *C. albicans* filamentation (9), we measured the pH of the supernatant medium. Interestingly, the pH of the supernatant isolated from *S.* Typhimurium grown in LB broth to late stationary phase was alkaline (pH 8.5). Furthermore, there was no difference in the antifungal activity of the *S.* Typhimurium supernatant after heat inactivation (100°C for up to 45 min).

***S.* Typhimurium inhibits *C. albicans* biofilm formation on silicone pads.** Given our observations of the effects of *S.* Typhimurium on *C. albicans* in the *C. elegans* coinfection model and under planktonic culture conditions in vitro, we assessed its effect on *C. albicans* biofilm formation on an abiotic surface (silicone pads). As shown in Fig. 5A, *S.* Typhimurium inhibited the development of *C. albicans* biofilm in a dose-dependent

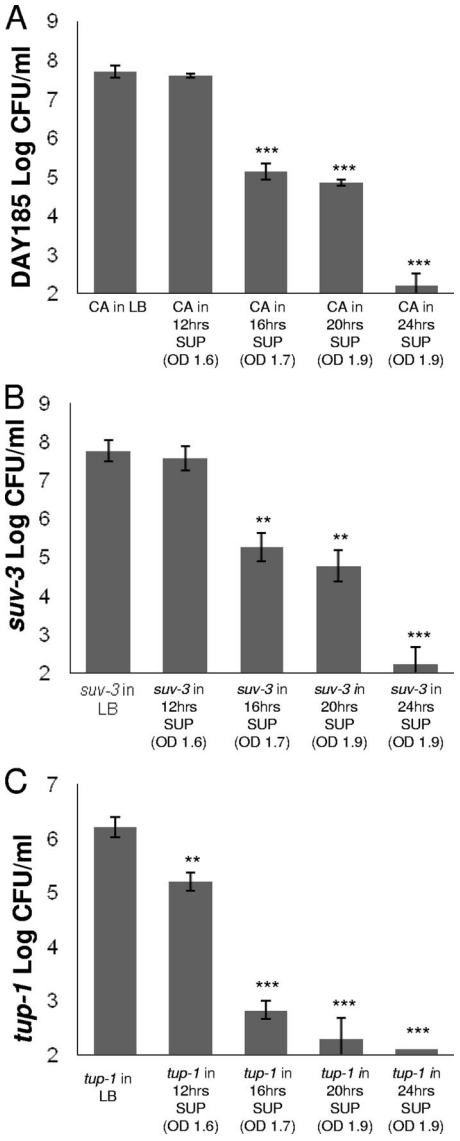

FIG. 4. *S.* Typhimurium supernatant (SUP) isolated at 37°C inhibits its *C. albicans* strain DAY185 (CA) in a growth-dependent fashion (A). Inhibition of *C. albicans* DAY185 (CA) and *suv3* mutant growth was not observed until they were exposed to supernatant taken from *S.* Typhimurium grown to late stationary phase (16 h, optical density at 600 nm [OD_{600}] of 1.7) (A and B, respectively). *tup1* mutant growth was inhibited in *S.* Typhimurium supernatant (SUP) isolated from early stationary phase (12 h, OD_{600} of 1) (C). When *C. albicans* strains DAY185 (CA) and *suv3* were grown in *S.* Typhimurium supernatant taken from a 24-h growth (OD_{600} of 1.9), the density of *C. albicans* was more than 7 log CFU/ml lower than that with growth in fresh LB medium (A and B). In the same supernatant, there was almost no growth for the *tup1* strain (C). All determinations of *C. albicans* CFU/ml were performed after 24 h of growth at 37°C. Column bars represent the means and error bars represent the standard deviations. Asterisks denote comparison of log CFU/ml with that of *C. albicans* in LB medium: ***, $P \leq 0.001$; **, $P \leq 0.01$ (two-tailed *t* test).

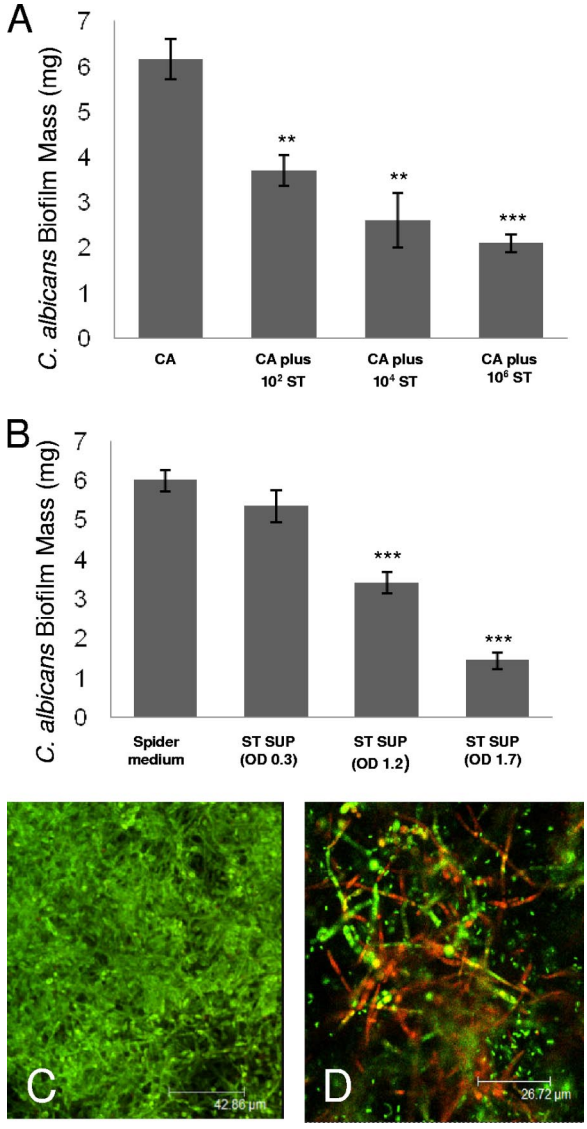

FIG. 5. *S.* Typhimurium (ST) inhibits the development of *C. albicans* (CA) biofilm on silicone pads. (A) The degree of inhibition of *C. albicans* (CA) biofilm formation was dependent on the density of *S.* Typhimurium (ST) inoculated into the biofilm environment. (B) *S.* Typhimurium supernatant (ST SUP) isolated at 37°C, when taken from a bacterial growth with an optical density (OD) at 600 nm of at least 1.2, led to a significant reduction in *C. albicans* biofilm mass compared to its development in fresh spider medium. (C and D) Fluorescent images of mature *C. albicans* biofilms (48 h of growth) when grown in the absence (C) or presence (D) of *S.* Typhimurium. with use of the Live/Dead staining system, nonviable (red stain) filaments are observed with incubation in the presence of *S.* Typhimurium. Column bars represent the means, and error bars represent the standard deviations. Asterisks denote comparison of *C. albicans* biofilm mass (mg) to *C. albicans* alone (A) or to *C. albicans* grown in spider medium (B): ***, $P \leq 0.001$; **, $P \leq 0.01$ (two-tailed *t* test). Scale bar: 42.86 μm (C) or 26.72 μm (D).

manner. A significant inhibition of *C. albicans* biofilm formation was observed after inoculation of as little as 10^2 CFU/ml of *S.* Typhimurium into the biofilm medium (spider medium) (Fig. 5A). Consistent with other results presented in this study (Fig. 1E and 4), *C. albicans* biofilm development was inhibited by filter-sterilized culture filtrate from *S.* Typhimurium (Fig. 5B). The degree of inhibition was dependent on the bacterial density in which the supernatant was derived (Fig. 5B). Confocal microscopy performed at the completion of the biofilm experiment (60 h) showed the presence of dead filaments with

incubation in the presence of *S.* Typhimurium compared to results in its absence (Fig. 5C and D). These data confirm that the antifungal properties of *S.* Typhimurium are effective toward *C. albicans* in a biofilm, a notoriously resistant environment.

Concluding remarks. In the present study, we have utilized the substitute host, *C. elegans*, to identify an antagonistic interaction between two human pathogens that reside within the gastrointestinal tract. We observed that *S.* Typhimurium inhibits its *C. albicans* filamentation, a key virulence determinant of this pathogen, in coinfection of *C. elegans*. Furthermore, after in vitro cocultures are performed in planktonic and biofilm environments, *S.* Typhimurium is capable of significantly reducing the viability of *C. albicans* and inhibiting its ability to form biofilm. Interestingly, culture filtrate from stationary-phase *S.* Typhimurium growth has significant anticandidal activity. The interaction of *C. albicans* with bacteria might shape microbial virulence and alter filamentation by *Candida* spp. in the intestinal tract. Identifying the molecular mechanisms of this interaction may provide important insights into microbial pathogenesis.

ACKNOWLEDGMENTS

Support was provided by National Institutes of Health R01 award AI075286 (to E.M.) and a University of Queensland postgraduate scholarship award (to A.Y.P.).

We thank Brian Ahmer for the *S.* Typhimurium *sdiA* mutant and the parent strain, 14028.

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