Killing of Candida albicans Filaments by Salmonella enterica Serovar Typhimurium Is Mediated by sopB Effectors, Parts of a Type III Secretion System\(^\text{\dag}\)\

Younghoon Kim and Eleftherios Mylonakis*\

Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, Massachusetts 02114\

Received 25 January 2011/Accepted 4 April 2011

Although bacterial-fungal interactions shape microbial virulence during polymicrobial infections, only a limited number of studies have evaluated this interaction on a genetic level. We report here that one interaction is mediated by sopB, an effector of a type III secretion system (TTSS) of Salmonella enterica serovar Typhimurium. In these studies, we screened 10 TTSS effector-related mutants and determined their role in the killing of C. albicans filaments in vitro during coinfection in planktonic environments. We found that deleting the sopB gene (which encodes inositol phosphatase) was associated with a significant decrease in C. albicans killing at 25°C after 5 days, similar to that caused by the deletion of sipB (which encodes TTSS translocation machinery components). The sopB deletion dramatically influenced the killing of C. albicans filaments. It was associated with repressed filamentation in the Caenorhabditis elegans model of C. albicans-S. Typhimurium coinfection, as well as with biofilm formation by C. albicans. We confirmed that SopB translocated to fungal filaments through SipB during coinfection. Using quantitative real-time PCR assays, we found that the Candida supernatant upregulated the S. Typhimurium genes associated with C. albicans killing (sopB and sipB). Interestingly, the sopB effector negatively regulated the transcription of CDC42, which is involved in fungal viability. Taken together, these results indicate that specific TTSS effectors, including SopB, play a critical role in bacterial-fungal interactions and are important to S. Typhimurium in order to selectively compete with fungal pathogens. These findings highlight a new role for TTSS of S. Typhimurium in the intestinal tract and may further explain the evolution and maintenance of these traits.

Microbial survival is based on diverse bacterial-bacterial, fungal-fungal, and bacterial-fungal interactions. These interactions are ubiquitous in nature, as well as in clinical environments, but very little is known about the genetic mechanism(s) associated with these interactions (51). Most of the previous studies have focused on Candida albicans, the opportunistic fungal pathogen that can exist as both yeast and filamentous cells according to its growing circumstances and conditions (59). C. albicans is the most common pathogenic fungus and may cause mucosal and systemic infections in immunosuppressed and immunocompetent hosts. The morphological transition from a yeast to a filamentous cell is critical for C. albicans pathogenesis (34, 55). Interestingly, the ability of C. albicans to develop filaments is also impacted by the presence of bacterial pathogens (8), and this association was extensively described during the interaction between C. albicans and various pathogenic bacteria, including Streptococcus gordonii (5), Staphylococcus epidermidis (2), Pseudomonas aeruginosa (25), Burkholderia cepacia (8), and Acinetobacter baumannii (52). However, limited work has been done on the interaction of C. albicans with intestinal bacterial pathogens. These studies are particularly important because in the human intestinal tract there exists a remarkable microbial community that includes C. albicans in essentially all humans (28, 51, 54).

Salmonella enterica serovar Typhimurium is one of the most common food-borne pathogens and can cause intestinal inflammation leading to diarrheal diseases in humans and animals (58). The type III secretion systems (TTSS) encoded by Salmonella pathogenicity island 1 (SPI-1) and SPI-2 on the bacterial chromosome are important virulence factors for Salmonella pathogenesis (74). Simply put, TTSS represents a molecular syringe allowing the bacteria to deliver effector proteins directly into the host cell cytosol (35, 74). To date, more than 30 SPI-1- and SPI-2-regulated effectors in Salmonella are known to use these systems to translocate these proteins into the host cell cytoplasm (37). Despite the importance of the TTSS in pathogenesis, the recognition and targeting of TTSS effectors remains poorly understood (17).

In previous work we found that the human intestinal pathogen S. Typhimurium significantly influenced the survival of C. albicans filaments (67). We found in the present study that the sopB effector is essential for competing with C. albicans filaments, and we show that S. Typhimurium influences the survival and filamentation of C. albicans in a Caenorhabditis elegans model and biofilm formation through the TTSS sopB effector. Moreover, the sopB effector can repress TEC1 (which encodes a transcription factor for filamentation and biofilm formation), HWPI and ALS3 (which encode filament specific cell wall proteins), and CDC42 (which encodes a Rho-type GTPase that is related to viability) in C. albicans.

MATERIALS AND METHODS

Fungal and bacterial strains, plasmids, and growth conditions. The strains and plasmids used in the present study are listed in Table 1. C. albicans and S. Typhimurium are commonly used food-borne pathogens and can cause intestinal infections in humans and animals (58). The type III secretion systems (TTSS) encoded by Salmonella pathogenicity island 1 (SPI-1) and SPI-2 on the bacterial chromosome are important virulence factors for Salmonella pathogenesis (74). Simply put, TTSS represents a molecular syringe allowing the bacteria to deliver effector proteins directly into the host cell cytosol (35, 74). To date, more than 30 SPI-1- and SPI-2-regulated effectors in Salmonella are known to use these systems to translocate these proteins into the host cell cytoplasm (37). Despite the importance of the TTSS in pathogenesis, the recognition and targeting of TTSS effectors remains poorly understood (17).

In previous work we found that the human intestinal pathogen S. Typhimurium significantly influenced the survival of C. albicans filaments (67). We found in the present study that the sopB effector is essential for competing with C. albicans filaments, and we show that S. Typhimurium influences the survival and filamentation of C. albicans in a Caenorhabditis elegans model and biofilm formation through the TTSS sopB effector. Moreover, the sopB effector can repress TEC1 (which encodes a transcription factor for filamentation and biofilm formation), HWPI and ALS3 (which encode filament specific cell wall proteins), and CDC42 (which encodes a Rho-type GTPase that is related to viability) in C. albicans.

*Corresponding author. Mailing address: Division of Infectious Diseases, Massachusetts General Hospital, Harvard Medical School, Boston, MA 02114. Phone: (617) 726-3812. Fax: (617) 726-7416. E-mail: emylonakis@partners.org.
†Published ahead of print on 15 April 2011.
TABLE 1. Strains and plasmids used in this study

| Strain or plasmid | Genotype and/or relevant characteristicsa | Source or reference |
|------------------|------------------------------------------|---------------------|
| **Bacterial strains** |                                           |                     |
| HB101            | Nonpathogenic E. coli; normal food source for C. elegans | 11                  |
| 1420S            | S. Typhimurium wild-type                  | ATCCa                |
| ∆sopB            | ∆sopB ΔKmr; Salmonella outer protein; homologue to ipgD of Shigella; regulated by SPI-1 | 58                  |
| ∆sopD            | ∆sopD ΔKmr; secreted protein in the Sop family; transferred to eukaryotic cells; regulated by SPI-1 | 58                  |
| ∆sopE2           | ∆sopE2 ΔKmr; type III secreted protein; regulated by SPI-1 | 58                  |
| ∆iptB            | ∆iptB ΔKmr; cell invasion protein; regulated by SPI-1 | 58                  |
| ∆invA            | ∆invA ΔKmr; invasion protein; regulated by SPI-1 | 58                  |
| ∆ssaE            | ∆ssaE ΔKmr; secretion system regulator; regulated by SPI-2 | 58                  |
| ∆ssE             | ∆ssE ΔKmr; secretion system regulator; regulated by SPI-2 | 58                  |
| ∆sso            | ∆sso ΔKmr; Salmonella translocated effector; regulated by SPI-2 | 58                  |
| ∆ladA           | ∆ladA ΔKmr; putative diguanylate cyclase/phosphodiesterase domain 1 | 58                  |
| ∆luxS           | ∆luxS ΔKmr; quorum-sensing protein, produces autoinducer signaling molecules | 58                  |
| ∆sphoP          | ∆sphoP ΔKmr; response regulator in two-component regulatory system | 58                  |
| ∆ssrA           | ∆ssrA ΔKmr; two-component regulatory system | 58                  |
| 1420S-GFP        | 1420S wild type containing pCM18          | This study           |
| ∆sopB-GFP       | ∆sopB containing pCM18                    | This study           |
| 1420S-SopB       | 1420S wild type containing pSB2908        | This study           |
| ∆sopB-SopB       | ∆sopB containing pSB2908                  | This study           |
| S. Typhimurium strains were routinely cultured in yeast-peptone-dextrose (YPD) and Luria-Bertani (LB) medium (Difco, Detroit, MI) at 37°C, respectively. When necessary, kanamycin (45 µg/ml), ampicillin (100 µg/ml), and erythromycin (300 µg/ml) were used for selective culture of mutants.

**Nematode strains.** C. elegans glp-4(mek2)sek(1)km4 strain was used for all experiments as described previously (11, 43). The C. elegans glp-4 mutant animals are defective for liquid assay experiments since worms are unable to produce progeny at 25°C; however, sterile animals have enhanced life span compared to wild-type animals (41). C. elegans sek-1 encodes a conserved mitogen-activated protein kinase kinase involved in innate immunity (29), expediting the time of progeny at 25°C; however, sterile animals have enhanced life span compared to wild-type animals (11). C. elegans strain N2 was used for all experiments. To quantify the viability of C. albicans in coinfection conditions, we used CFU analysis as previously described (22, 25, 67). YPD agar plates containing kanamycin (45 µg/ml), ampicillin (100 µg/ml), and streptomycin (100 µg/ml) were used for selective culture of C. albicans strains. The CFU were determined by diluting cells by 10^3 to 10^7 via 10-fold serial dilution steps in 0.85% NaCl solution that was applied as 10-µl drops on agar plates (14). The YPD agar plates were incubated at 30°C for 48 h. Two independent cultures were used for each strain.

**In vitro coinfection assay under planktonic environments.** In vitro coculture assays were performed in 2 ml of LB broth and incubated in a roller drum at 25°C for 5 days (52). A starting inoculum of ca. 10^6 CFU/ml of S. Typhimurium strain 14208 wild type or its isogenic mutants (58) (Table 1) and ca. 5 × 10^6 cells/ml of C. albicans DAY185 were used for all experiments. To quantify the viability of C. albicans in coinfection conditions, we used CFU assay as previously described (22, 25, 67). YPD agar plates containing kanamycin (45 µg/ml), ampicillin (100 µg/ml), and streptomycin (100 µg/ml) were used for selective culture of C. albicans strains. The CFU were determined by diluting cells by 10^3 to 10^7 via 10-fold serial dilution steps in 0.85% NaCl solution that was applied as 10-µl drops on agar plates (14). The YPD agar plates were incubated at 30°C for 48 h. Two independent cultures were used for each strain.

**In vitro coinfection assay for filament specific killing.** In order to monitor the killing and inhibition of C. albicans filaments via the sopB effector, we evaluated C. albicans filamentation from C. albicans ∆sop1 and C. albicans SSOY50-B. C. albicans ∆sop1 is a strain that constitutively produces filaments at 25°C, while the ∆sop2 mutant produces no filaments under the same conditions (10, 55). In addition, we studied the genetically engineered strain C. albicans SSOY50-B (tetacycline-regulatable tet-NRG1) that constitutively forms filaments in the presence of 20 µg of doxycycline (DOX)/ml at 37°C but does not produce filaments in the absence of DOX (59). Using these strains, we evaluated the viability of C. albicans filaments infected with S. Typhimurium strains by using the XTT assay (2,3-bis-(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) assay as described earlier (38) with slight modifications. More specifically, C. albicans ∆sop1 and ∆sop2 were cultured at 25°C for 48 h in order to produce filaments and yeast cells, respectively. In addition, C. albicans SSOY50-B was grown at 37°C for 24 h with or without 20 µg of DOX/ml to facilitate the formation of filaments and yeast-type cells, respectively. Filaments and normal yeast cells were washed three times with phosphate-buffered saline (PBS) and incubated with 10^6 CFU of S. Typhimurium/ml in LB medium for 24 h. After incubation, the C. albicans filaments were recovered by centrifugation at 5,000 rpm for 3 min, washed three times by PBS, and then the XTT-menadione solution, consisting 10 ml of XTT solution (10 mM menadione in acetone) was added. After incubation for 2 h at 37°C and centrifugation, 80 µl of the XTT-menadione supernatants was transferred to the wells of a 96-well microtiter plate, and measured by using a microtiter plate reader ( Molecular Devices, Sunnyvale, CA) at 490 nm. All results were normalized based on the E. coli HB101 control.

**C. elegans coinfection assay for filamentation.** The C. elegans coinfection assays for C. albicans filamentation were performed as previously described (67). In brief, synchronized, young adult nematodes were preinfected for 4 h on lawns of C. albicans DAY185 and then transferred into wells of a six-well microtiter dish (40 worms per well), followed by three washes with M9 medium. Each well contained 2 ml of liquid assay medium (20% brain heart infusion and 80% M9). S. Typhimurium strains were inoculated at the concentration of ca. 10^6 CFU/ml before the addition of the C. albicans-infected worms. The plates were then incubated at 25°C and examined at 24-h intervals for 6 days for viability and the formation of penetrative filaments by using a Nikon SMZ645 dissecting microscope. In addition, to evaluate whether Salmonella strains influence filament elongation of C. albicans in C. elegans, worms with initial filamentation (worms...
with filaments after 1 day) were moved to new wells of 96-well plates (Corning no. 3882), including S. Typhimurium strains expressing green fluorescent protein (GFP) (Table 1; ca. 10^6 CFU/ml) in liquid assay medium and then incubated at 25°C for an additional 5 days. The qualitative observation of C. albicans filamentation and elongation in C. elegans was performed by using a Discovery-1 microscope (Molecular Devices, Sunnyvale, CA) using a fluorescein isothiocyanate (FITC) filter set or with bright-field transmitted light.

5. Typhimurium attachment to C. albicans filaments. To evaluate the filamentation in vitro, C. albicans SSYS0-B (50) organisms were inoculated into YPD with 20 μg of DOX/ml in 96-well plates (Corning, Inc., Corning, NY; no. 3882) and incubated for 8 h at 37°C with shaking at 150 rpm. After incubation, the wells were washed five times with PBS, and ca. 10^6 CFU/ml of Salmonella strains expressing GFP in LB containing erythromycin (300 μg/ml) were added, followed by incubation for 15 h at 37°C. After six washings with PBS to remove unattached Salmoneella strains, GFP-expressing Salmoneella strains on C. albicans filaments were observed by using a Discovery-1 microscope under FITC and bright-field light. In addition, S. Typhimurium cells attached to C. albicans filaments were counted by using the CFU assay, following recovery of C. albicans filaments from 96-well plates using vigorous pipetting. LB agar plates containing fluconazole (32 μg/ml) were used to select for S. Typhimurium. Plates were incubated at 37°C for 24 h.

Silicone pad biofilm assay. The effect of S. Typhimurium on C. albicans biofilm growth was evaluated by using a polychromic silicone pad assay as described previously (52). Spider medium (32) 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 control silicone pads exposed without fungal cells.

Monitoring of SopB in C. albicans. To monitor the SopB delivered into fungal filaments from S. Typhimurium, filamentous C. albicans SSYS0-B cells (i.e., the genetically engineered Candida strain with constitutive filaments under DOX) cultured in the presence of 20 μg of DOX/ml were collected with wild type or the S. Typhimurium sipB mutant (TTSS translocation machinery component deficient) expressing FLAG epitope-tagged SopB (50) under 0.015% L-arabinose for 15 h. After gentamicin treatment (100 μg/ml) for 1 h to kill exterior filament-binding Salmoneella, fungal cells were pelleted for immunofluorescence by using monoclonal anti-FLAG M2 FITC antibody (Sigma-Aldrich Corp., St. Louis, MO) based on the manufacturer’s protocol. Fluorescent observation of SopB in filaments was performed by using confocal laser microscopy (TCS-NT; Leica Microsystems).

qRT-PCR. Quantitative reverse transcription-PCR (qRT-PCR) was performed using the CHROMO4 real-time PCR system (MJ Research, Inc., Weltham, MA). After disruption with glass beads (Sigma-Aldrich), total RNA was isolated according to the protocol of an RNeasy minikit (Qiagen, Valencia, CA), including an on-column DNase digestion with RNase-free DNase (Qiagen). After the RNA was isolated, 50 ng of total RNA was used for the qRT-PCR using a SuperScript III Platinum SYBR Green One-Step qRT-PCR kit (Invitrogen, Carlsbad, CA). Primers were designed by using Primer3Input Software (v0.4.0; http://frodo.wi.mit.edu/primer3/) and are listed in Table 2. Relative expression levels were calculated by using the 2^(-ΔΔCt) method (35). The control genes 18S rRNA and 16S rRNA were used to normalize the expression data for Candida and Salmonella, respectively. The annealing temperature was 60°C for all of the genes in the present study. To investigate the transcriptional regulations of sopB and sipB by C. albicans supernatants, overnight cultures of C. albicans DAY185 ΔsopB versus ΔsopB (at 25°C) and C. albicans SC5314 versus ΔsopB/ΔsopB (at 37°C) were inoculated in YPD and cultured for 48 and 24 h, respectively. Supernatants were quickly recovered, filtered, and stored at −80°C before use. The sopB and sipB transcript levels were investigated after exposure to prepared supernatants from C. albicans or farnesol (Sigma-Aldrich; 100 and 200 μM concentrations) for 2 h. In contrast, C. albicans DAY185 filamentous cells on silicone pads (18 h) were exposed to the S. Typhimurium wild type and mutants for 3 h. After infection by the Salmoneella strains, the C. albicans filamentous cells on pads were washed twice with PBS and then quickly recovered by vigorous pipetting. The results were normalized using the E. coli strain HB101, which has no impact on C. albicans filamentation and biofilm formation.

Statistical analysis. C. elegans survival was examined by using the Kaplan-Meier method, and differences were determined by using the log-rank test (STATA6; STATA. College Station, TX). Differences in the number from each experiment were determined by using a Student t test. Each result is a representative experiment of at least two independent biological replicates. A P value of 0.05 in all replicate experiments was considered statistically significant.

---

### TABLE 2. Oligonucleotides used for qRT-PCR

| Organism and gene | Orientation | Sequence (5’–3’) |
|-------------------|-------------|------------------|
| Salmonella sopB   | R           | CTATACAACGGGAATCGAGATTCTC AGTTGATGGGTGCATAGGGTCTTTG |
| sipB              | F           | ATTACTGCTTGGCAGAATGTTAGGCTC TTCAGACCTGATGTCATAGGTT |
| Candida TCE1      | F           | TGGTGCTTATTCCAGGTCCTC TTCTGAATTTCCGCGTTTGT |
| HWPJ              | F           | CCTTACGCGTCTCAGAGTTGTT TGGCAGAATGGTTGCTAGAT |
| ALS3              | R           | ACTCTCAAGACGTCCTCCAC TCGAATGGCAGCTACCA |
| CDC42             | F           | AGGGGTAATGTTGCTAAGGAGA TGCAGGTCATATAGTTGCA |
| 18S rRNA          | F           | GTGCCAGCAGCGCGGGTA TGGACGCGGCAGCGACG |

*a F, forward primer; R, reverse primer.*

---

### RESULTS

Role of SPI-1 effectors in the viability of C. albicans. In order to evaluate the hypothesis that TTSS is involved in the S. Typhimurium-C. albicans interaction, we selected 10 mutant strains that have a mutation involving different TTSS effectors. They can be categorized into three groups: (i) strains with a mutation involving the SPI-1 system effectors, including SopB (48), SopD (69), and SopE2 (4); (ii) strains with a mutation involving the SPI-2 system effectors, including SsaE (40), SsbE (23), and SscJ (49); and (iii) strains with a mutation involving TTSS-related regulators such as A DrA (18), LuxS (66), PhoP (20), and SsrA (7). Note of is that there was no significant difference in the growth rates in vitro among these S. Typhimurium mutants (data not shown).

Using this collection of S. Typhimurium strains, we compared the viability of C. albicans exposed to the S. Typhimurium wild-type (WT) strain or isogenic mutants using in vitro confection assays. At 25°C after 5 days, the deletion of sopB strongly enhanced the survival of C. albicans by 16.6-fold compared to the WT strain (Fig. 1). In contrast, deletion of SPI-2 gene ssaE, sseB, or sscJ or global regulator gene adrA, luxS, phoP, or ssrA still repressed the fungal viability similar to the WT. Previous reports demonstrated that translocases (including SipB) controlled by SPI-1 are required for translocation of SopB into the host cell cytosol (71). To explore whether the SipB translocation machinery component is also linked to killing of C. albicans, we examined the viability of C. albicans coinfectected with the sipB mutant. Consistent with the results observed with the sopB mutant, the viability of C. albicans was also increased as a result of the sipB deletion (16.8-fold; Fig. 1).

To summarize the analysis of the S. Typhimurium mutant strains, the sopB effector and the sipB translocase influence the anticanidal activity of the bacterium.
The \textit{sopB} effector selectively represses the viability of \textit{C. albicans}. Gram-negative bacteria, such as \textit{A. baumannii} (52) and \textit{P. aeruginosa} (25) bind to and kill \textit{C. albicans} filaments without affecting yeast cells, and our group showed that \textit{S. Typhimurium} may preferentially kill filamentous \textit{C. albicans} cells over yeast cells (67). However, the viability measured using CFU assays does not accurately reflect the number of viable cells for filamentous fungi (9). Therefore, in order to evaluate the effect of the \textit{sopB} effector against \textit{C. albicans} filaments, we also used XTT assays (38). As expected, \textit{C. albicans} \textit{ΔsopB} cells grown under conditions that promote filament development were highly susceptible to \textit{S. Typhimurium} WT, whereas deleting \textit{sopB} and \textit{sipB} limited the effect of \textit{S. Typhimurium} (Fig. 2A). Consistently, we verified a similar activity using the \textit{C. albicans} SSY50-B filaments (Fig. 2B). Importantly, the effect was limited in yeast-type cells from \textit{C. albicans} \textit{ΔsuV3} or from strain SSY50-B grown without DOX (Fig. 2). These results indicate that the \textit{sopB} effector is important for the killing of \textit{C. albicans} filaments.

\textit{SopB} is translocated into filaments of \textit{C. albicans} through \textit{SipB}. To visualize the translocation of \textit{SopB} into filaments, \textit{C. albicans} cells were coinfected with \textit{S. Typhimurium} strains expressing FLAG-tagged \textit{SopB} that enable immunodetection of the protein (Table 1). We found that the fluorescent \textit{SopB} protein shifts into the interior of the \textit{C. albicans} filaments; however, there was no signal or \textit{SopB} translocation when we used the \textit{sipB} mutant (merged images in Fig. 3). In addition, we detected weak fluorescent \textit{SopB} signals in yeast-type cells (data not shown). This finding demonstrates that, similar to what happens during the interaction of mammalian cells with \textit{S. Typhimurium} (50), the \textit{SopB} effector protein translocates into fungal filaments via \textit{SipB}.

\textit{sopB} is essential for the attachment to \textit{C. albicans} filaments. We hypothesize that the \textit{sopB} effector can also influence \textit{S. Typhimurium} attachment to \textit{C. albicans} filaments, since SPI-1 effectors have been shown to be required for \textit{Salmonella} attachment to eukaryotic host cells (31). To evaluate this hypothesis, we investigated the attachment of \textit{S. Typhimurium} WT and the \textit{sopB} mutant to filaments produced by \textit{C. albicans}. In this experiment, \textit{C. albicans} SSY50-B filaments were prepared in the presence of 20 \textmu g of DOX/ml and then added to either the GFP-expressing \textit{S. Typhimurium} WT or the \textit{sopB} mutant. As expected, fluorescent GFP intensity was decreased in the \textit{sopB} deletion mutant on \textit{C. albicans} filaments compared to the WT (Fig. 4A). Moreover, by direct CFU counting, we verified that the deletion of either \textit{sopB} or \textit{sipB} resulted in a significant reduction in bacterial attachment to \textit{C. albicans} filaments consistently by >10-fold, and this repression was abolished by complementation of \textit{SopB} (Fig. 4A; \textit{P} < 0.05). These results suggest that, at least in part, the \textit{sopB} effector share the function for attachment on fungal filaments with mammalian cells.

\textit{The \textit{sopB} effector mediates inhibition of \textit{C. albicans} filaments in vivo.} To explore whether \textit{sopB} plays a direct role in the inhibition of \textit{C. albicans} filaments in vivo, we utilized the \textit{C. elegans} coinfection assay. Using this assay we previously reported that \textit{S. Typhimurium} (but not nonpathogenic \textit{E. coli} strains) can inhibit \textit{C. albicans} filamentation (67), even though \textit{S. Typhimurium} alone is toxic in \textit{C. elegans} (data not shown). Corroborating the viability tests, deleting \textit{sopB} and \textit{sipB} significantly increased the number of filament-coated worms compared to the WT (Fig. 4B), but filamentation in the presence of the \textit{sopB} mutant was less than that observed for \textit{E. coli} HB101 or \textit{C. albicans} alone (\textit{P} < 0.05). Corroborating these results, we
found that the number of worms with filaments was restored by complementing SopB. This finding using the model host *C. elegans* indicates that the *sopB* effector is an important factor that inhibits *C. albicans* filaments in *vivo*.

We also evaluated whether the *sopB* effector is involved in the inhibition of preformed filaments. After the forming filaments of *C. albicans* in nematodes for 1 day, we transferred *C. elegans* into fresh liquid medium containing the GFP expressing *S. Typhimurium* WT and the *sopB* mutant (Table 1). Consistently, we found that exposure to *S. Typhimurium* WT inhibits filament elongation, but the effect of the *sopB* mutant was significantly less (Fig. 4C). In addition, we introduced GFP-expressing *Salmonella* strains to visualize the binding of *S. Typhimurium* to *C. albicans* filaments and inhibit their elongation (Fig. 4C, white arrow). We found that, similar to our *in vitro* studies reported above, *sopB* is essential for the association of *S. Typhimurium* with *C. albicans* filaments. Moreover, as in the *in vitro* and *in vivo* studies detailed above, our findings also indicated that *sopB* is essential for the initiation of the fungal germ tubes (Fig. 4D). Hence, we conclude that the *sopB* effector is directly involved in an antagonistic effect against *C. albicans* filaments at different stages of hyphal development.

**The *sopB* effector mediates the *S. Typhimurium* effect on *C. albicans* biofilm.** The morphological transition from yeast to filaments is a major requirement for biofilm formation, as well as virulence (16, 55). Therefore, we considered the possibility that the *sopB* effector may influence *C. albicans* biofilm formation. This hypothesis is an extension of our finding above in which *S. Typhimurium* attaches to filaments (Fig. 4A), and this inhibited filament formation and elongation (Fig. 4B and C) and selectively killed *C. albicans* filaments (Fig. 1 and 2) via the *sopB* effector. We evaluated the effect of *S. Typhimurium* on the *C. albicans* biofilm using silicone pads (67). As shown in Fig. 4E, the robust *C. albicans* biofilm was dramatically repressed by the *S. Typhimurium* WT. This profound reduction was depleted when the *C. albicans* biofilm was exposed to the *sipB* or *sopB* mutants (*P* < 0.05 compared to the WT), while they were still statistically different with the biofilms of *C. albicans* alone. Accordingly, complementing *SopB* restored the inhibition of biofilm formation. Our observations confirm that the killing of filaments mediated by *sopB* effector affects filamentation, as well as fungal biofilm formation.

**The *S. Typhimurium* *sopB* effector negatively regulates the transcription of *TEC1, HWP1, ALS3, and CDC42* in *C. albicans*.** Using qRT-PCR, we found that *sopB* is upregulated by supernatant obtained from *C. albicans* filaments [Fig. 5A; (2.5 ± 0.4)-fold in Δ*up1* versus Δ*sw3* strains and (8.9 ± 0.3)-fold in SC5314 versus Δ*cpH1*Δ*efg1* strains]. Also, under these conditions, *sipB* was slightly induced by the supernatant from filamentous *C. albicans* cells. At least in part, these findings are in agreement with our results on the role of *sopB* and *sipB* in competing *C. albicans* filaments (Fig. 1). We further evaluated whether the *C. albicans* quorum-sensing signal farnesol influenced the regulation of *sopB* and *sipB*, but farnesol at 100 and 200 μM did not alter the transcription of either of these genes (data not shown).

Initially, we hypothesized that *S. Typhimurium* TTSS effectors, including *SopB* and *SipB*, regulate specific components associated with the *C. albicans* morphological transition. Preliminary results among essential virulence factors, including *NRG1* (which encodes a transcriptional repressor and regulates filament formation and virulence) (44), *TUP1* (which encodes a transcriptional corepressor and represses filamentous growth) (10), *TEC1* (which encodes a TEA/ATTS transcription factor involved in the pheromone response pathway in white cells and the regulation of filament-specific genes) (60), *EFG1* (which encodes a transcriptional repressor) (64), *HWP1* (which encodes a filament-specific cell wall protein) (47), *ALS3* (which encodes a filament-specific adhesin) (72), *SOD2* (which encodes superoxide dismutase) (27), and *CDC42* (which encodes a Rho-type GTPase) (56), indicate that *S. Typhimurium* supernatants selectively repressed transcriptional levels of *TEC1, HWP1, ALS3*, and *CDC42* (data not shown). Moreover, we investigated whether the transcription
of TECl, HWP1, ALS3, and CDC42 genes in C. albicans are influenced by the direct contact between the fungus and S. Typhimurium and the translocation of the sopB effector. The qRT-PCR assay showed that the transcriptional levels of TECl [(−5.9 ± 0.3)-fold], HWP1 [(−7.1 ± 1.6)-fold], and ALS3 [(−5.6 ± 0.3)-fold], were significantly repressed by S. Typhimurium WT, whereas these reductions were restored by the deletion of sopB effector or the sipB translocase gene (Fig. 5B). It is reasonable that the function of TECl, HWP1, and ALS3 is linked to hyphal development and biofilm formation in C. albicans as described previously (47, 60, 72). More importantly, the transcription of CDC42 was strongly decreased by (10.4 ± 0.6)-fold in the presence of S. Typhimurium. Critically, CDC42 is essential for Candida viability (39). Therefore, our qRT-PCR results suggest that, at least in part, the sopB effector translocated into filaments through...
SipB may kill *C. albicans* filaments through associating with *CDC42*.

**DISCUSSION**

Bacterial-fungal encounters are common in nature, as well as in clinical settings, and shape microbial pathogenesis. The interactions between fungal and bacterial pathogens have been previously extensively investigated (2, 5, 8, 25, 52, 67). Of importance is the diverse population of bacteria and fungi that coexist within the human intestinal tract. However, very little is known about the specific mechanisms underlying these interactions. In the present study we demonstrate that TTSS and especially the *sopB* effector are essential for *S. Typhimurium* to survive the interaction with intestinal fungi, such as *C. albicans*. The role of the *sopB* effector, as well as that of the associated translocase SipB, is based on the following five findings. (i) The deletion of *sopB* significantly increased the survival of *C. albicans in vitro* (Fig. 1). (ii) The *sopB* effector translocates into filaments via SipB (Fig. 3) and kills *C. albicans* filaments (Fig. 2). (iii) Similar to the findings in *vitro*, *sopB* of *S. Typhimurium* diminished the viability of *C. albicans* filaments during *C. elegans* infection (Fig. 4). (iv) The *sopB* effector repressed the elongation of filaments and germ tubes, as well as *Candida* biofilm formation (Fig. 4). (v) Remarkably, the *sopB* effector is associated with the transcriptional repression of *CDC42* in *C. albicans* (Fig. 5). Based on these findings, we report that the *sopB* effector of *S. Typhimurium* is an important weapon for competing against fungi, and this constitutes a novel role for bacterial TTSS effectors.

We recently utilized *C. elegans* to study monomicrobial infection due to *C. albicans* (11, 53) or *S. Typhimurium* (1), as well as to study the *C. albicans*-*S. Typhimurium* coinfection (67). This model of intestinal infection is particularly relevant for the study of microbial infections (45). In our previous observation (67), the *S. Typhimurium-C. albicans* competition in polymicrobial infection seemed to be mediated by direct adhesion to filaments, as well as growth-dependent molecules secreted by the bacteria. Therefore, we investigated the hypothesis that TTSS effectors may play a role in *S. Typhimurium* competition against *C. albicans*. The TTSS apparatus and its effector proteins are exclusively expressed and secreted at the stationary phase of growth (61) and are strongly linked to virulence traits, such as contact, invasion, and biofilm formation (37). Interestingly, we found that the antagonistic interaction between *S. Typhimurium* and *C. albicans* is multifactorial and that the viability of *C. albicans* is associated with the *S. Typhimurium sopB* and *sipB* TTSS translocation machinery units (Fig. 1).

We show that the TTSS genes regulated by SPI-1 (including *sopB* and *sipB*) are involved in competing with fungal pathogens. TTSS that are regulated by SPI-1 mainly mediate the initial attachment of *S. Typhimurium* on mammalian cells and the initiation of mammalian cell death is SPI-1 dependent (30), whereas the SPI-2 component of TTSS involve postinvasion processing, including vacuole maturation (15). Importantly, *sopB* and *sipB* are associated with SPI-1 regulation. The *Salmonella* SopB (also known as SigD) from *Shigella flexneri* (46) and is an inositol phosphate that acts on the phospholipids in the host cell membrane (30, 57). SopB is the SPI-1-regulated effector for TTSS that is quickly translocated into the mammalian host after contact (50) and is linked to enteropathogenesis of mammalian cells since *sopB* defective mutants of *Salmonella* are attenuated (48). Therefore, we suggest that SopB may modify membrane phospholipids of *C. albicans* filaments and that this modification results in filament death. In addition, SipB is required for the assembly of the TTSS needle complex and is one of the effectors regulated by SPI-1. Interestingly, SipB helps the translocation of SPI-1 effectors, including SopB into host cells (71, 74), and triggers mammalian cell death directly (24). Of note is that the regulatory system encoded by *ssrA*, which controls the SPI-2 system (7), had no effect in the competition with *C. albicans* (Fig. 1). Consistent with these observations, the *sopB* effector is essential for the *S. Typhimurium* attachment to filaments of *C. albicans* (Fig. 4A). Furthermore, after translocating into the filaments via the SipB translocase (Fig. 3), the *sopB* effector can inhibit filament elongation (Fig. 4C) in *C. elegans*, as well as fungal biofilm formation on silicone squares (Fig. 4E).

Generally, bacteria, including *S. Typhimurium*, produce quorum-sensing (QS) signaling molecules to regulate expression of a number of genes, including the *luxA* gene, which is important for the synthesis of this QS signal in *S. Typhimurium* (66). Importantly, the QS signaling molecule is also resistant to acid and heat (65). Boone et al. (8) identified a novel signaling molecule in *B. cenocepacia* that is a structural homologue of a QS molecule that inhibited germ tube and filament formation of *C. albicans*. Thus, we postulated that a QS-related gene is
also essential in interacting with fungi. Unexpectedly, in the present study, the deletion of luxS had no impact on the viability of C. albicans (Fig. 1), which was also true with another other QS sensor gene saxA that did not inhibit C. albicans filaments (65). Of note is that the activity of this signal molecule is maximal at mid-exponential phase and dramatically decreases at the stationary phase (65). Although bacterial QS signals were not responsible for the antifungal effects of S. Typhimurium on C. albicans directly, previous work has shown that 3-oxo-C12 homoserine lactone, the QS signaling molecule produced by P. aeruginosa, was involved in C. albicans filamentation (26), and our group has reported that QS is involved in the Acinetobacter-Candida interaction (52). These results suggest that different strategies to fight C. albicans may be dependent on the specific genus of bacteria.

Even though TTSS-related components were not directly linked in the Pseudomonas-Candida interaction, previous reports demonstrate that effector proteins such as ExoS are toxic in Saccharomyces cerevisiae (63) and clearly contribute to the toxicity of Pseudomonas toward amoebae (36). Importantly, it has been reported that SopB strongly influences the viability of S. cerevisiae (3). Interestingly, ExoS and SopB share a function in mammals by modulating GTPase and actin polymerization (3, 36). Importantly, we show that the sopB effector strongly repressed CDC42 mRNA levels (Fig. 5B). Notably, silencing of CDC42 is lethal in Candida (39) and inhibits the growth of mammalian cells (70). More importantly, Cdc42p (cell division cycle 42) is involved in C. albicans filament-specific characteristics, including hyphal growth and filament-specific gene transcripts (12, 68, 73). A recent report describes that the SopB effector protein strongly binds with the small G-protein Cdc42p (12, 68, 73). Interestingly, Cdc42p is also required for invasion via hyphal growth of C. albicans (6), our future goal is to establish the pathway that inhibits or kills filaments through the SopB effector-Cdc42p association.

We also report the observation that the C. albicans supernatant induces sopB transcripts, whereas the sopB effector repressed TEC1, HWPI, and ALS3 transcripts (Fig. 5). It was extensively established that filamentation and biofilm formation are involved in C. albicans virulence and that TEC1, HWPI, and ALS3 are important key genes for the hyphal development and biofilm formation of C. albicans (47, 60, 72). Equally important, other investigators confirmed that deletion of TEC1 and HWPI resulted in a significant reduction of C. albicans virulence in Galleria mellonella (16) and mice (62). Moreover, our qRT-PCR results are consistent with our biofilm data that the C. albicans biofilm was critically repressed by the sopB effector (Fig. 4E). Therefore, these results indicate that in addition to its effect in mammals, SopB may influence the transcription of genes involved in hypha- and biofilm-associated fungal pathogenesis during killing events.

Bacterial-fungal interactions play a significant role in human health since they may enhance, modulate, or decrease microbial pathogenesis (42). Intestinal bacteria need to overcome the other intestinal microflora, and the interaction of C. albicans with intestinal Gram-negative bacteria may control filamentation. The sopB effector plays a critical role in allowing the bacteria to attach and kill C. albicans filaments, and the viability and filamentation of C. albicans is influenced by sopB. Our working model suggests that in the presence of C. albicans filaments, the sopB effector is activated. The SopB effector translocates into the fungal filaments via SipB translocale and clearly kills filaments. This killing event may trigger the repression of biofilm formation. Our results propose a novel role for TTSS effectors in bacterial-fungal interactions.

**ACKNOWLEDGMENTS**

This study was supported by grants from the National Institutes of Health (P01 AI083214, R01 AI075286, and R21 AI079569) to E.M. We are grateful for the pSB2908 and pCM18 plasmids provided by Jorge E. Galán (Yale University) and Thomas K. Wood (Texas A&M University), respectively. We thank Michael McClelland (Sidney Kimmel Cancer Center) for providing the S. Typhimurium 14028 wild-type and the isogenic mutants. We also thank Beth B. Fuchs for help with the confocal microscopy and Jeffrey J. Coleman for useful discussions.

**REFERENCES**

1. Abalay, A., and F. M. Ausubel. 2001. Programmed cell death mediated by ced-3 and ced-4 protects Caenorhabditis elegans from Salmonella typhimurium-mediated killing. Proc. Natl. Acad. Sci. U. S. A. 98:2733–2739.

2. Adam, R., G. S. Raiblle, and I. J. Douglas. 2002. Mixed species biofilms of Candida albicans and Staphylococcus epidermidis. J. Med. Microbiol. 51:334–349.

3. Alemán, A., et al. 2005. The amino-terminal non-catalytic region of Salmonella typhimurium SigD affects actin organization in yeast and mammalian cells. Cell Microbiol. 7:1432–1436.

4. Bakshi, C. S., et al. 2000. Identification of SopE2, a Salmonella secreted protein which is highly homologous to SopE and involved in bacterial invasion of epithelial cells. J. Bacteriol. 182:2341–2344.

5. Bamford, C. V., et al. 2009. Streptococcus gordonii modulates Candida albicans biofilm formation through intergeneric communication. Infect. Immun. 77:3906–3904.

6. Bassilana, M., J. Hopkins, and R. A. Arkowitz. 2005. Regulation of the Cdc42/Cdc24 GTase module during Candida albicans hyphal growth. Europatol. Cell 4:588–603.

7. Beazer, L., et al. 2008. The Salmonella pathogenicity island 2 regulator sopA promotes reproductive trait but not intestinal colonization in chickens. Vet. Microbiol. 126:216–224.

8. Boone, C., et al. 2008. A novel DSF-like signal from Burkholderia cenocepacia interferes with Candida albicans morphological transition. ISME J. 2:29–36.

9. Bowman, J. C., et al. 2001. Quantitative PCR assay to measure Aspergillus fumigatus burden in a murine model of disseminated aspergillosis: demonstration of efficacy of caspofungin acetate. Antimicrob. Agents Chemother. 45:3474–3481.

10. Braun, B. R., and A. D. Johnson. 1997. Control of filament formation in Candida albicans by the transcriptional repressor TUP1. Science 277:105–109.

11. Breger, J., et al. 2007. Antifungal chemical compounds identified using a Caenorhabditis elegans pathogenicity assay. PLoS Pathog. 3:e18.

12. Court, H., and P. Sudbery. 2007. Regulation of Cdc42 GTase activity in the formation of hyphae in Candida albicans. Mol. Biol. Cell 18:261–268.

13. Davis, D., R. B. Wilson, and A. P. Mitchell. 2000. RIM101-dependent and-independent pathways govern pH responses in Candida albicans. Mol. Cell. Biol. 20:971–978.

14. Donegan, K., C. Matyac, R. Seidler, and A. Porteous, 1991. Evaluation of methods for sampling, recovery, and enumeration of bacteria applied to the phylloplane. Appl. Environ. Microbiol. 57:51–56.

15. Drecksch, D., L. A. Knodler, K. Galbraith, and O. Steele-Mortimer. 2005. The Salmonella SPI1 effector SopB stimulates nitric oxide production long after invasion. Cell Microbiol. 7:105–113.

16. Fuchs, B. R., et al. 2010. Role of filamentation in Galleria mellonella killing by Candida albicans. Microbes Infect. 12:488–496.

17. Galán, J. E., and H. Wolf-Watz. 2006. Protein delivery into eukaryotic cells by type III secretion machines. Nature 444:567–573.

18. García, B., et al. 2004. Role of the GGDEF protein family in Salmonella cellulose biosynthesis and biofilm formation. Mol. Microbiol. 54:264–277.

19. Gillum, A. M., E. Y. Tsay, and D. R. Kirsch. 1984. Isolation of the Candida albicans gene for orotidine-5’-phosphate decarboxylase by complementation of Saccharomyces cerevisiae ura3 and Escherichia coli pyrF mutations. Mol. Gen. Genet. 198:179–182.

20. Groisman, E. A., E. Chiao, C. J. Lipps, and F. Heffron. 1989. Salmonella
typhimurium phoP virulence gene is a transcriptional regulator. Proc. Natl. Acad. Sci. U. S. A. 96:7077–7081.

21. Hansen, M. C., R. J. J. Palmer, C. Udsen, D. C. White, and S. Molin. 2001. Assessment of GFP fluorescence in cells of Streptococcus gordonii under conditions of low pH and low oxygen concentration. Microbiology 147:1383–1391.

22. Harrriott, M. M., and M. C. Noverr. 2010. Ability of Candida albicans mutants to induce Staphylococcus aureus vancomycin resistance during polymicrobial biofilm formation. Antimicrob. Agents Chemother. 54:3754–3755.

23. Hensel, M., et al. 1998. Genes encoding putative effector proteins of the type III secretion system of Salmonella pathogenicity island 2 are required for bacterial virulence and proliferation in macrophages. Mol. Microbiol. 30:173–184.

24. Hersh, D., et al. 1999. The Salmo nella invasin InvP induces macrophage apoptosis by binding to caspase-1. Proc. Natl. Acad. Sci. U. S. A. 96:2396–2401.

25. Hogan, D. A., and R. Kolter. 2002. Pseudomonas-Candida interactions: an ecological role for virulence factors. Science 296:2229–2232.

26. Hogan, D. A., A. Vik, and R. Kolter. 2004. A Pseudomonas aeruginosa quorum-sensing molecule influences Candida albicans morphology. Mol. Microbiol. 54,42–122.

27. Iwamoto, S. Y., U. Bask, H. S. Yim, and S. O. Kang. 2003. Protective roles of mitochondrial manganese-containing superoxide dismutase against various stresses in Candida albicans. Yeast 20:929–941.

28. Keeny, K. M., and B. B. Finlay. 2011. Enteric pathogen exploitation of the microbiota-generated nutrient environment of the gut. Curr. Opin. Microbiol. 14:92–98.

29. Kim, D. H., et al. 2002. A conserved p38 MAP kinase pathway in Caenorhabditis elegans innate immunity. Science 297:623–626.

30. Knodler, L. A., B. B. Finlay, and O. Steele-Mortimer. 2005. The Salmo nella effector protein SpolP protects epithelial cells from apoptosis by sustained activation of Akt. J. Biol. Chem. 280:9058–9064.

31. Lara-Tejero, M., and J. E. Galán. 2009. Salmonella enterica serovar typhi murium pathogenicity island 1-encoded type III secretion system translocons mediate intimate attachment to nonphagocytic cells. Infect. Immun. 77:2635–2642.

32. Liu, H., J. Köhler, and G. R. Fink. 1999. Suppression of hyphal formation in Saccharomyces cerevisiae by phosphatase-dead SigD/SopB from Salmonella typhimurium. Microbiology 152:3437–3452.

33. Rogers, L. D., et al. 2008. Identification of cognate host targets and specific ubiquitination sites on the Salmonella SPI-1 effector SopB. J. Proteomics 71:97–108.

34. Song, M., Z. L. Li, L. D. Li, and H. Wang. 2006. Inhibition of Cdc42-dependent signaling in Caenorhabditis elegans by phosphatase-dead SseJ. Microbiology 152:2273–2285.

35. Schweizer, A., S. Rupp, B. N. Taylor, M. Röllinghoff, and K. Schröppel. 2009. The TEA/ATTS transcription factor CaTe1p regulates hyphal development and virulence in Candida albicans. Mol. Microbiol. 38:435–445.

36. Song, M., et al. 2004. ppGpp-dependent stationary phase induction of genes on Salmonella pathogenicity island 1. J. Biol. Chem. 279:24183–24190.

37. Staal, J. F., S. D. Bradway, P. L. Fidel, and P. F. Siodsun. 1996. Adhesive and mammalian transglutaminase substrate properties of Candida albicans Hwp1. J. Biol. Chem. 271:1535–1538.

38. Stirling, F. R., and T. J. Evans. 2006. Effects of the type III secreted pseudomonal toxin Etxs in the yeast Saccharomyces cerevisiae. Microbiology 152:2273–2285.

39. Stoldt, V. R., A. Sonneborn, C. E. Leuker, and J. F. Ernst. 1997. Eligip, an essential regulator of morphogenesis of the human pathogen Candida albicans, is a member of a conserved class of BHLH proteins regulating morphogenetic processes in fungi. EMBO J. 16:1982–1991.

40. Surette, M. G., and B. L. Bassler. 1998. Quorum sensing in Escherichia coli. Annu. Rev. Microbiol. 52:697–730.

41. Surette, M. G., B. L. Bassler, and B. L. Bassler. 1999. Quorum sensing in Escherichia coli, Salmonella typhimurium, and Vibrio harveyi: a new family of genes responsible for autoinducer production. Proc. Natl. Acad. Sci. U. S. A. 96:13631–13636.

42. Tampakakis, E., A. Y. Peleg, and E. Mylonakis. 2009. Interaction of Candida albicans with an intestinal pathogen, Salmonella enterica serovar Typhimurium. Eukaryot. Cell 8:732–737.

43. VandenBerg, A. L., A. S. Ibrahim, J. E. Edwards, K. A. Toenjes, and D. Johnson. 2010. Cotton2p GTase regulates the budding-to-budded-form transition and expression of hypha-specific transcripts in Candida albicans. Eukaryot. Cell 9:575–589.

44. Wood, M. W., et al. 2004. Structural analysis of Salmonella serovar Typhimu rium SPI-1 effector protein. Mol. Microbiol. 54:1217–1224.

45. Wu, F., et al. 2007. RNA-interference-mediated Cdc42 silencing downregulates phosphorylation of STAT3 and suppresses growth in human bladder cancer cells. Biotechnol. Appl. Biochem. 49:121–128.

46. Zhang, S., et al. 2002. The Salmonella enterica serovar typhimurium effector proteins SipA, SopA, SopB, and SopE2 set in concert to induce diaphragm in calves. Infect. Immun. 70:3843–3855.

47. Zhao, X., et al. 2006. Candida albicans Als0p is required for wild-type biofilm formation on silicone elastomer surfaces. Microbiology 152:2287–2293.

48. Zheng, X. D., R. T. Lee, Y. M. Wang, Q. S. Lin, and Y. Wang. 2007. Phosphorylation of Rga2, a Cdc42 GAP, by Cdk1/Hck1 is crucial for Candida albicans hyphal growth. EMBO J. 26:3760–3769.

49. Zhou, D., and J. Galán. 2001. Salmonella entry into host cells: the work in concert of type III secreted effector proteins. Microbes Infect. 3:1293–1298.