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Killing of *Candida albicans* Filaments by *Salmonella enterica* Serovar Typhimurium Is Mediated by *sopB* Effectors, Parts of a Type III Secretion System[▽]

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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 cenocepacia* (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), *HWP1* 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.*

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TABLE 1. Strains and plasmids used in this study

Strain or plasmid	Genotype and/or relevant characteristics[a]	Source or reference
Bacterial strains		
HB101	Nonpathogenic *E. coli*; normal food source for *C. elegans*	11
14028	*S.* Typhimurium wild-type	ATCC[b]
Δ*sopB*	Δ*sopB* Ω Km^r; *Salmonella* outer protein; homologue to *ipgD* of *Shigella*; regulated by SPI-1	58
Δ*sopD*	Δ*sopD* Ω Km^r; secreted protein in the Sop family; transferred to eukaryotic cells; regulated by SPI-1	58
Δ*sopE2*	Δ*sopE2* Ω Km^r; type III secreted protein effector; regulated by SPI-1	58
Δ*sipB*	Δ*sipB* Ω Km^r; cell invasion protein; regulated by SPI-1	58
Δ*invA*	Δ*invA* Ω Km^r; invasion protein; regulated by SPI-1	58
Δ*ssaE*	Δ*ssaE* Ω Km^r; secretion system effector; regulated by SPI-2	58
Δ*sseB*	Δ*sseB* Ω Km^r; secretion system effector; regulated by SPI-2	58
Δ*sseJ*	Δ*sseJ* Ω Km^r; *Salmonella* translocated effector; regulated by SPI-2	58
Δ*adrA*	Δ*adrA* Ω Km^r; putative diguanylate cyclase/phosphodiesterase domain 1	58
Δ*luxS*	Δ*luxS* Ω Km^r; quorum-sensing protein, produces autoinducer signaling molecules	58
Δ*phoP*	Δ*phoP* Ω Km^r; response regulator in two-component regulatory system	58
Δ*ssrA*	Δ*ssrA* Ω Km^r; two-component regulatory system	58
14028-GFP	14028 wild type containing pCM18	This study
Δ*sopB*-GFP	Δ*sopB* containing pCM18	This study
14028/SopB	14028 wild type containing pSB2908	This study
Δ*sipB*/SopB	Δ*sipB* containing pSB2908	This study
Δ*sopB*/SopB	Δ*sopB* containing pSB2908	This study
Candida strains		
DAY185	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 his1*::*hisG*/*HIS1*::*his1*::*hisG arg4*::*hisG*/*URA3*::*ARG4*::*arg4*::*hisG*	13
Δ*tup1*	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 his1*::*hisG*/*tup1*::*hisG-URA3-hisG*	10
Δ*suv3*	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 suv3*::Tn*7-URA3*/*suv3*::*Tn7-UAU1 his1*::*hisG*/*his1*::*hisG arg4*::*hisG*/*arg4*::*hisG*	55
SC5314	Clinical isolate	19
Δ*cph1*/Δ*efg1*	*ura3*Δ::λ*imm434*/*ura3*Δ::λ*imm434 cph1*Δ::*hisG*/*cph1*Δ::*hisG efg1*Δ::*hisG*/*efg1*Δ::*hisG-URA3-hisG*	34
SSY50-B	*ura3*Δ::λ*imm434*/*tet-NRG1*/*URA3*	59
Plasmids		
pCM18	Em^r; pTRKL2-P_{CP25}-RBSII-*gfpmut3*; broad host	21
pSB2908	Am^r; pBAD24 P_{lac}::FLAG-tagged $SopB^+$; arabinose inducible	50

[a] Am^r, Em^r, and Km^r represent ampicillin, erythromycin, and kanamycin resistance, respectively.
[b] ATCC, American Type Culture Collection.

Typhimurium strains were routinely cultured in yeast-peptone-dextrose (YPD) at 30°C 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*(*bn2*);*sek-1*(*km4*) strain was used for all experiments as described previously (11, 43). The *C. elegans glp-4* mutant animals are suited 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 experiments. Worms were cultured and maintained on *Escherichia coli* HB101 by using standard procedures (11).

***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 14028 wild type or its isogenic mutants (58) (Table 1) and ca. 5×10^5 cells/ml of *C. albicans* DAY185 were 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 to select for *C. albicans* strains. The CFU were determined by diluting cells by 10^0 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* filaments from *C. albicans* Δ*tup1* and *C. albicans* SSY50-B. *C. albicans* Δ*tup1* is a strain that constitutively produces filaments at 25°C, while the Δ*suv3* mutant produces no filaments under the same conditions (10, 55). In addition, we studied the genetically engineered strain *C. albicans* SSY50-B (tetracycline-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 [2,3-*bis*-(2-methoxy-4-nitro-5-sulfophenyl)-2*H*-tetrazolium-5-carboxanilide] assay as described earlier (38) with slight modifications. More specifically, *C. albicans* Δ*tup1* and Δ*suv3* were cultured at 25°C for 48 h in order to produce filaments and yeast cells, respectively. In addition, *C. albicans* SSY50-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 (0.5 mg/ml in PBS) and 1 μl of menadione (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.

***S.* Typhimurium attachment to *C. albicans* filaments.** To evaluate the filamentation *in vitro*, *C. albicans* SSY50-B (59) 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 *Salmonella* strains, GFP-expressing *Salmonella* 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 polymicrobial 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* SSY50-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 coinfected with wild type or the *S.* Typhimurium *sipB* mutant (TTSS translocation machinery component deficient) expressing FLAG epitope-tagged SopB (50) under 0.05% L-arabinose for 15 h. After gentamicin treatment (100 μg/ml) for 1 h to kill exterior filament-binding *Salmonella*, fungal cells were processed 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., Waltham, 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 $2^{-\Delta\Delta CT}$ method (33). 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 Δ*tup1* versus Δ*suv3* (at 25°C) and *C. albicans* SC5314 versus Δ*cph1*/Δ*efg1* (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 *Salmonella* 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[a]	Sequence (5′–3′)
Salmonella		
sopB	F	CTTATACAACGGAATGCAGATTCTC
	R	AGTTATAGAGGTTATGCAGCGAGTG
sipB	F	ATTACTGCTTGGCAAGTTAATGACC
	R	TTTGATACTGGCTTCATAGAGATCC
16S rRNA	F	TGTAGCGGTGAAATGCGTAG
	R	CAAGGGCACAACCTCCAAG
Candida		
TEC1	F	TGGTGCTTATTCACGTGTCC
	R	TTCTGAATTTCCCGGTTTTG
HWP1	F	CTCCAGCTGGCTCAAGTGGT
	R	TGGCAGATGGTTGCATGAGT
ALS3	F	ACTTCCACAGCTGCTTCCAC
	R	TGCAGATGGAGCATTACCAC
CDC42	F	AGGGTGAAAAATTGGCTAAGGA
	R	TGCAGCTACTATAGCCTCGTCA
18S rRNA	F	GTGCCAGCAGCCGCGGTA
	R	TGGACCGGCCAGCCAAGC

[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), SseB (23), and SseJ (49); and (iii) strains with a mutation involving TTSS-related regulators such as AdrA (18), LuxS (66), PhoP (20), and SsrA (7). Of note 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* coinfection conditions. 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 *sseJ* 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* coinfected 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 anticandidal activity of the bacterium.

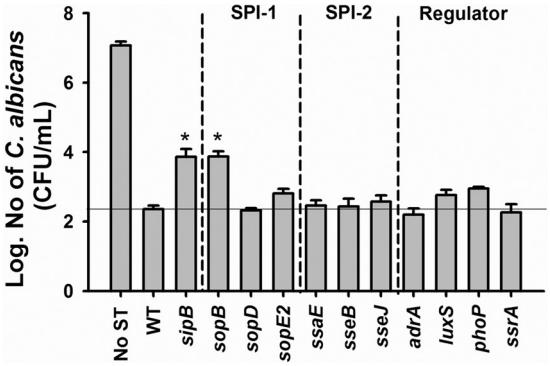

FIG. 1. The *S.* Typhimurium *sopB* effector influences the viability of *C. albicans* under an *in vitro* planktonic environment. The viability of *C. albicans* in the presence of *S.* Typhimurium wild type (WT) or various mutants was determined at 25°C after 5 days. The error bars represent the standard errors of the mean for two independent biological replicates. Asterisks indicate significantly different values ($P < 0.05$; Student *t* test).

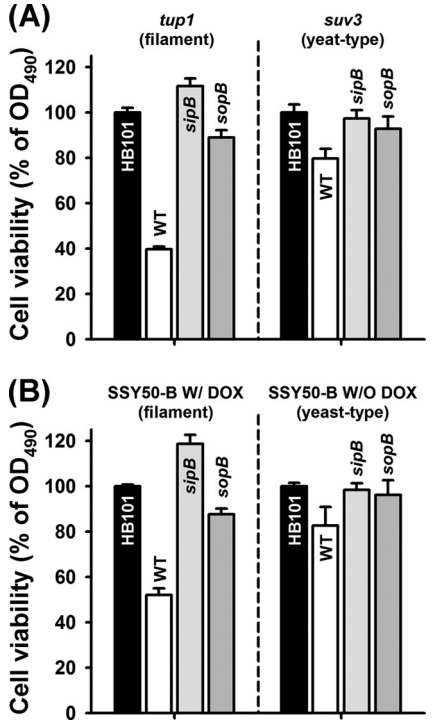

FIG. 2. *S.* Typhimurium kills *C. albicans* filaments via the *sopB* effector. Viability of *C. albicans* filaments using the XTT assay. (A) *C. albicans* Δ*tup1* or Δ*suv3* strains were cultured at 25°C for 48 h for constitutive filament production or normal yeast cells, respectively. (B) In addition, the experiments with *C. albicans* SSY50-B were performed in the presence or absence of DOX (20 μg/ml) for abundant filaments or yeast form cells. Filaments or yeast cells were exposed to *S.* Typhimurium wild-type (WT) and mutant strains for 24 h. The cell viability was evaluated and normalized against the HB101 control. Bars represent the standard errors of the mean for two independent biological replicates.

The *sopB* effector selectively represses the viability of *C. albicans*. Gram-negative bacteria, such as *A. baumannii* (52) and *P. aeruginosa* (25) bind to and kill *C. albicans* filaments without affecting yeast cells, and our group showed that *S.* Typhimurium may preferentially kill filamentous *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 *sopB* effector against *C. albicans* filaments, we also used XTT assays (38). As expected, *C. albicans* Δ*tup1* cells grown under conditions that promote filament development were highly susceptible to *S.* Typhimurium WT, whereas deleting *sopB* and *sipB* limited the effect of *S.* Typhimurium (Fig. 2A). Consistently, we verified a similar activity using the *C. albicans* SSY50-B filaments (Fig. 2B). Importantly, the effect was limited in yeast-type cells from *C. albicans* Δ*suv3* or from strain SSY50-B grown without DOX (Fig. 2). These results indicate that the *sopB* effector is important for the killing of *C. albicans* filaments.

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

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

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

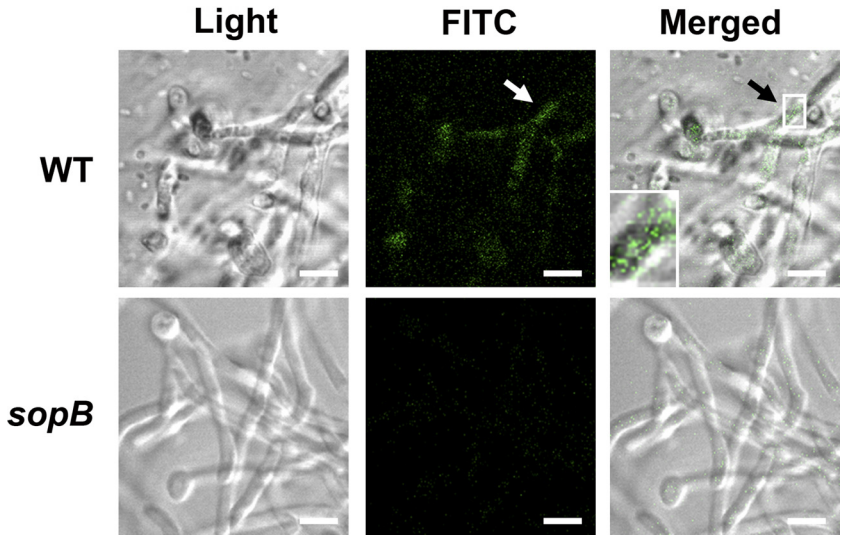

FIG. 3. Translocation of SopB into *C. albicans* filaments via SipB. SopB translocation in *S.* Typhimurium wild-type (top row) and *sipB* mutant (bottom row) was visualized by confocal microscopy. Scale bars, 4.2 μm. The inset shows an enlarged view of the area indicated by the arrow. An immunofluorescence assay was performed as described in Materials and Methods.

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 medicated 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*tup1* versus Δ*suv3* 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 adhesion) (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

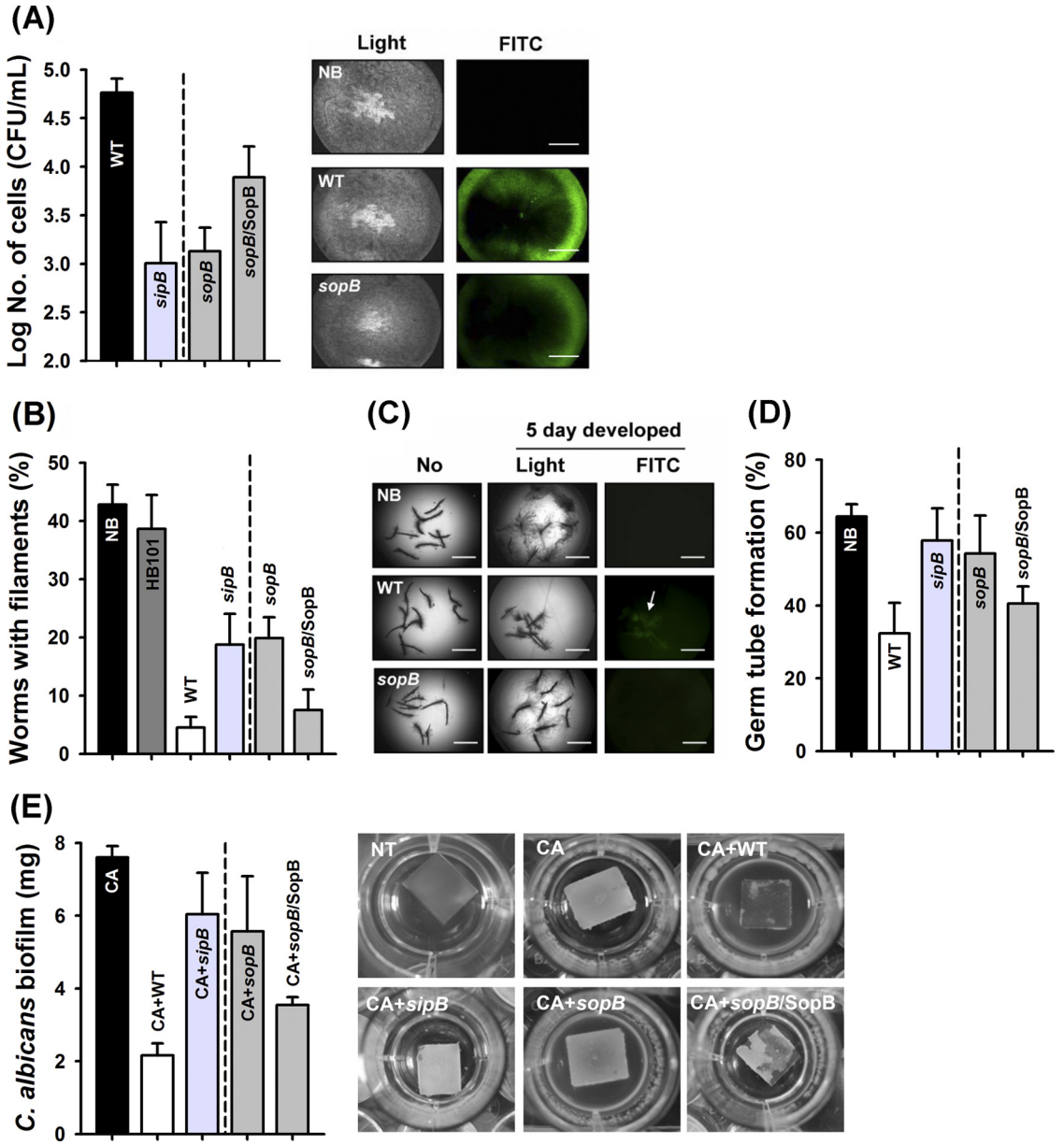

FIG. 4. *S.* Typhimurium strongly inhibits *C. albicans* filamentation and biofilm formation via the *sopB* effector. (A) Attachment of *S.* Typhimurium wild-type (WT) and *sopB* mutant strains to the *C. albicans* filaments. *C. albicans* SSY50-B filaments were formed in 96-well plates with 20 μg of DOX/ml and infected with GFP-expressing *Salmonella* strains for 15 h at 37°C. *Salmonella* attachment on *C. albicans* filaments was visualized by using fluorescence microscopy (scale bars, 1 mm) and counted by using the CFU assay. (B) Inhibition of *C. albicans* DAY185 filamentation in the *C. elegans* coinfection model. (C) Repression of filament elongation in the *C. elegans* coinfection model. An arrow indicates the GFP expression of *S.* Typhimurium cells on the *C. albicans* filaments (scale bars, 1 mm). (D) Inhibition of germ tube formation. (E) *C. albicans* biofilm formation on silicone squares in spider medium for 60 h at 37°C (NT, no treatment; CA, *C. albicans* alone; CA+WT, *C. albicans* cocultured with *S.* Typhimurium wild type; CA+*sopB* or *sipB*, *C. albicans* cocultured with the *S.* Typhimurium *sopB* or *sipB* mutants; CA+*sopB*/SopB, *C. albicans* cocultured with the *S.* Typhimurium *sopB* mutant overproducing SopB).

of *TEC1*, *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 *TEC1* [(−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 *TEC1*, *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

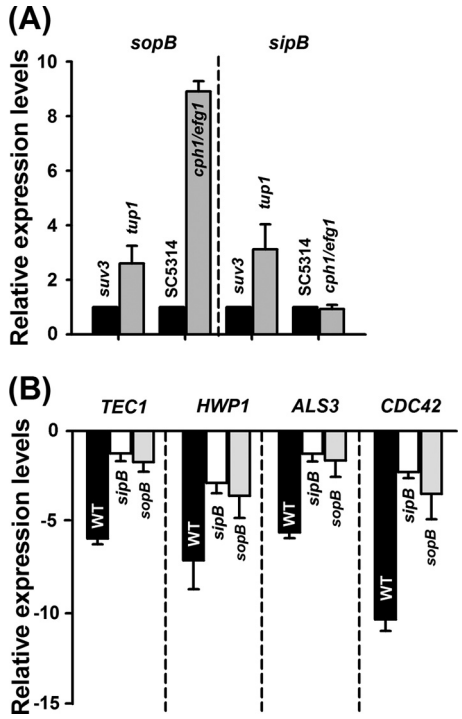

FIG. 5. Gene expression in *S.* Typhimurium and *C. albicans*. (A) Transcription of *sopB* and *sipB* of the *S.* Typhimurium wild type in the presence of supernatants from filaments or yeast-form *C. albicans*. (B) Transcriptional levels of *TEC1*, *HWP1*, *ALS3*, and *CDC42* in the presence or absence of *sopB* or *sipB*. The fold change was normalized by comparison to an HB101 control.

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) is a homologue of IpgD 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 *luxS* 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 *sdiA* 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-C_{12} 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 in yeast (56), as well as in mammalian cells (57). Therefore, it seems reasonable to assume that, at lease in part, the SopB effector of *S.* Typhimurium affects *C. albicans* filaments through association with Cdc42p. In addition, because, 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*, *HWP1*, and *ALS3* transcripts (Fig. 5). It was extensively established that filamentation and biofilm formation are involved in *C. albicans* virulence and that *TEC1*, *HWP1*, 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 *HWP1* 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 transcriptions 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 translocase 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.

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